UNIVERSITAT DE BARCELONA

Intensive exercise and immunity: effect of diets enriched in fibre and/or flavonoids in rats

Patricia Ruiz Iglesias

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Facultat de Farmàcia i Ciències de l'Alimentació
Departament de Bioquímica i Fisiologia
Secció de Fisiologia

INTENSIVE EXERCISE AND IMMUNITY: EFFECT OF DIETS ENRICHED IN FIBRE AND/OR FLAVONOIDS IN RATS

Patricia Ruiz Iglesias

Barcelona, 2022

UNIVERSITAT DE BARCELONA

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INTENSIVE EXERCISE AND IMMUNITY: EFFECT OF DIETS ENRICHED IN FIBRE AND/OR FLAVONOIDS IN RATS

Memòria presentada per Patricia Ruiz Iglesias per optar al títol de doctor amb menció internacional per la Universitat de Barcelona.

Aquesta tesi ha estat realitzada al grup d'*Autoimmunitat, Immunonutrició i Tolerància* sota la supervisió de la Dra. Margarida Castell Escuer i el Dr. Francisco José Pérez Cano. La doctoranda ha realitzat una estada de recerca al *Nutrition-Gut-Brain Interactions Research Centre de la Universitat d'Örebro*, Suècia, sota la supervisió del Dr. Robert Jan Brummer.

Dra. Margarida Castell Escuer	Dr. Francisco José Pérez Cano	Patricia Ruiz Iglesias
(directora i tutora de la tesi)	(director de la tesi)	(doctoranda)

PATRICIA RUIZ IGLESIAS
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Grup d'Autoimmunitat, Immunonutrició i Tolerància
Departament de Bioquímica i Fisiologia
Facultat de Farmàcia i Ciències de l'Alimentació
Edifici B, 3a planta
Av. Joan XXIII, 27-31, 08028 Barcelona
Tel. +34 934 024 505

Dra. Margarida Castell Escuer, professora catedràtica, i Dr. Francisco J. Pérez Cano, professor titular, de la Secció de Fisiologia del Departament de Bioquímica i Fisiologia de la Facultat de Farmàcia de la Universitat de Barcelona,

INFORMEN

Que la memòria titulada "*Intensive exercise and immunity: effect of diets enriched in fibre and/or flavonoids in rats"* presentada per PATRICIA RUIZ IGLESIAS per optar al Títol de Doctor amb Menció Internacional per la Universitat de Barcelona, ha estat realitzada sota la nostra direcció a la Secció de Fisiologia del Departament de Bioquímica i Fisiologia i, considerant-la conclosa, autoritzem la seva presentació per ser jutjada pel tribunal corresponent.

I perquè així consti, signem el present,

Barcelona, 1 de juliol de 2022

Dra. Margarida Castell Escuer
Secció de Fisiologia
Departament de Bioquímica i Fisiologia
Facultat de Farmàcia i Ciències de l'Alimentació
Universitat de Barcelona

Dr. Francisco J. Pérez Cano
Secció de Fisiologia
Departament de Bioquímica i Fisiologia
Facultat de Farmàcia i Ciències de l'Alimentació
Universitat de Barcelona

ABSTRACT

The influence of exercise on immune system has gained increasing attention from researchers lately, mainly due to the growing participation of the general population in sport competitions. Whereas moderate intensity exercise enhances immune function, high intensity exercise can have a deleterious effect on the immune system, which, in addition to increase the risk of infections, is associated with a decline in exercise performance and an impaired recovery. Furthermore, intensive exercise can disrupt the gastrointestinal integrity and function, which results in the appearance of symptoms of varying severities. Nevertheless, the mechanisms underlying these gastrointestinal effects remain unclear. It seems that splanchnic ischaemia, altered motility, malabsorption and neuroendocrine factors may be involved, although the exercise-induced immune alterations may also play an important role. Nutritional strategies aimed at counteracting these effects are needed. Previous studies have shown the immunomodulatory, antioxidant and anti-inflammatory properties of polyphenols, especially of those found in cocoa and oranges.

Considering this background, the main goals of the current thesis were to establish the immune alterations induced by intensive exercise on the gut microbiota composition and the mucosal immune system and to evaluate the possible preventive properties of cocoa, cocoa fibre and hesperidin on such alterations, focusing on the changes induced in mucosal immunity as well as those induced in systemic immunity

To achieve the initial part of the thesis goal, two different training protocols were used. First, to assess changes in caecal microbiota composition, male and female Wistar rats performed two 30-min running sessions per day for 15 days followed by a final exhaustion test (ET). Although these training programme slightly modified the gut microbiota composition in both male and female Wistar rats, most of the alterations depended on the rats' sex. After this first approach, a more intense exercise model was used in Wistar rats for assessing the exercise-induced alterations in mucosal immunity; the model included three trainings and two exhaustion tests per week, for 5 weeks. This longer training programme was able to disrupt the mucosal immunity, by reducing salivary IgA and altering the composition and function of mesenteric lymph nodes lymphocytes, as well as the intestinal epithelial barrier integrity, by altering the gene expression of tight junction proteins.

To accomplish the second part of the objective, female Wistar rats were fed either a standard diet, a diet containing 10% cocoa providing 5% fibre, or a diet containing only

5% cocoa fibre. After 25 days of dietary intervention, half of the rats of each diet performed an exhaustion test without prior training, given that acute exercise also impairs immune system. Both cocoa- and cocoa fibre-enriched diets protected against the oxidative stress induced by a single session of exhausting exercise, although they differently modulated the mucosal immunity.

We next established the effects of supplementation with a pure flavonoid, hesperidin, in the immune alterations induced by the 5-week training programme. Hesperidin enhanced the exercise performance and prevented the increase in circulating leukocytes induced by the final exhaustion test, as well as the higher secretion of IFN-γ by peritoneal macrophages induced by training. After observing these promising results, a dietary intervention with both cocoa and hesperidin was also evaluated. In this case, Lewis rats undertook a 6-week intensive training programme in an uphill treadmill. Although some preventing effects after the dietary interventions were found, such as avoiding the plasma cortisol increase and some of the immune alterations induced by exercise, such effects were mainly associated with cocoa.

Overall, different models of intensive exercise have been applied and evidenced some immune impairments. Dietary interventions with cocoa, cocoa fibre and hesperidin have partially prevented these changes, without affecting exercise performance, except for the ergogenic effects induced by oral supplementation with hesperidin.

CONTENTS

INTRODUCTION 1
1. EXERCISE AND IMMUNE SYSTEM 1
1.1 Exercise intensity and immune function 1
1.2 Exercise and circulating leukocytes 4
1.3 Exercise and innate effector cells 5
1.3.1 Granulocytes 6
1.3.2 Monocytes and macrophages 7
1.3.3 NK cells 8
1.4 Toll-like receptors 9
1.5 Systemic adaptive immunity 11
1.5.1 Lymphocyte redistribution 11
1.5.2 Lymphocyte function 12
1.5.2.1 *Lymphocyte proliferation* 13
1.5.2.2 *Cytokine secretion and Th1/Th2 balance* 13
1.5.2.3 *Immunoglobulin production* 14
1.6 Exercise and the mucosa-associated lymphoid tissue 15
1.7 Exercise, gastrointestinal ischemia and intestinal barrier function 17
1.8 Exercise and gut microbiota 19
2. FLAVONOIDS AND IMMUNE SYSTEM 21
2.1 Cocoa 21
2.1.1 Cocoa composition 22
2.1.2 Antioxidant properties of cocoa 22
2.1.3 Anti-inflammatory properties of cocoa 23
2.1.4 Cocoa as immunomodulator 25
2.1.4.1 *Effects on lymphocyte composition and function* 26
2.1.4.2 *Effects on humoral immune response* 27
2.1.4.3 *Gut microbiota* 30
2.2 Hesperidin 31
2.2.1 Antioxidant properties of hesperidin 32
2.2.2 Anti-inflammatory effects of hesperidin 33
2.2.3 Hesperidin as immunomodulator 34

3. EXERCISE, FLAVONOIDS AND IMMUNE SYSTEM 35
Review article 37
OBJECTIVES 79
RESULTS 83
Article 1 85
Article 2 95
Article 3 113
Article 4 131
Article 5 153
Article 6 175
DISCUSSION 207
CONCLUSIONS 217
REFERENCES 221

INTRODUCTION

1. EXERCISE AND IMMUNE SYSTEM

According to the World Health Organization (WHO), physical activity refers to any movement of the body produced by skeletal muscles that involves energy expenditure (1). When the practice of physical activity is planned, organised and repetitive for the purpose of improving body function and performance, it is called exercise.

It is well known that moderate intensity exercise offers several long-term health benefits. The WHO recommends the practice of at least 150-300 minutes per week of moderate intensity aerobic exercise (1). The regular practice of exercise decreases the risk of cardiovascular and metabolic diseases (2–4), and it can prevent, delay or even improve the prognosis of several chronic inflammatory diseases (5–7) and even cancer (8,9). However, when the exercise practice is overly intense or there is not a prior adequate training, it can induce adverse effects on health (10), such as oxidative stress (11), inflammation (12) and muscle damage (13), as well as immune (14) and gastrointestinal (GI) alterations (15).

1.1 EXERCISE INTENSITY AND IMMUNE FUNCTION

The relationship between exercise and immune function started to be studied at the beginning of the 20th century, when changes in white blood cell differential counts were observed in Boston marathon runners (16). In the 1980s, several epidemiological studies reported an association between the practice of exhausting exercise and a higher risk of upper respiratory tract infections (URTIs) during the two following weeks (17). However, exercise immunology as a discipline started to gain momentum in 1989 with the foundation of the International Society of Exercise Immunology and the *Exercise Immunology Review* journal (17,18). In 1994, Nieman draw the hypothesis of the “J” curve model for representing the relationship established between exercise intensity and the disease susceptibility (19), and in 1999, Woods *et al.* adapted this model adding an “inverted J” for representing the association between exercise intensity and immune function (**Figure 1**) (20). These models indicate that whereas regular bouts of moderate exercise enhance immune function, overly intensive exercise may impair it, decreasing host protection accordingly and leading to a higher risk of infections, especially of those affecting the mucosa such as URTIs and gastrointestinal GI infections. These transitory suppressions of the immune system normally lasts from 3 to 72 h, although it could be even longer if the recovery periods between intensive bouts are not respected, and it may open a window for opportunistic infections (the

open-window hypothesis) (14,21,22). Apart from the appearance of symptomatology of varying severity (common cold, acute sinusitis, acute pharyngitis, etc.) (23), the existence of a prerace infection has also been associated with a lower exercise performance or even a higher risk of not finishing the competition (17,24).

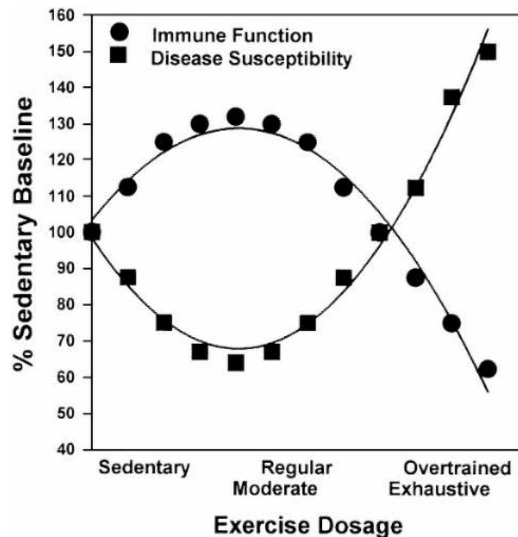

Figure 1. Model proposed by Woods *et al.* about the changes induced by different intensities of exercise in the immune function and the disease susceptibility (20). Adaptation of the J-curve model previously proposed by Nieman (19).

The exercise practice induces a transient inflammatory status and affects both the innate and the adaptive immune function, depending the outcome on the intensity and duration of the effort (**Figure 2**). Innate immune system is the first line of defence against invading pathogens and includes physical barriers, microbiota, non-specialized effector cells and cell receptors, as well as antimicrobial peptides and soluble mediators. Both the acute and the chronic practice of intensive exercise alter the number and function of neutrophils, macrophages and natural killer (NK) cells, which classically belong to the innate immune system (see section 1.2.2 for further details) (25). Moreover, exercise has also shown to affect the intestinal epithelial barrier (26,27) and the concentration of salivary antimicrobial peptides such as α-defensins, lactoferrin and lysozyme (28–30). On the other hand, the adaptive immune response is characterized by the involvement of antigen-specific lymphocytes and antibodies, and the generation of immunological memory. Exercise can modify the function of both T and B lymphocytes, altering the production of immunoglobulins (Ig) (see section 1.5.2.3 for further details), especially in the mucosal compartment, as well as the secretion of cytokines (see section 1.5.2.2 for further details).

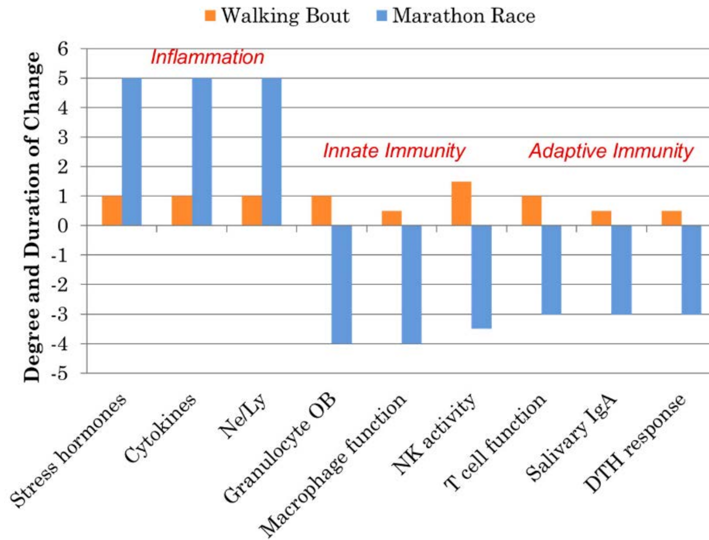

FIGURE 2. Comparison of the immune responses to a moderate intensity exercise (30- to 45-min walking bout) and a heavy exertion (marathon race). NK = natural killer; Ne/Ly = ratio of neutrophil to lymphocyte cell counts; OB = oxidative burst activity; NK = natural killer; DTH = delayed-type hypersensitivity. Image from Nieman *et al.* (14).

Not only the intensity and duration of exercise are important when considering its effects on the immune function, but also the recovery periods between trainings and intensive bouts (**Figure 3**). When there is a proper balance between trainings and adequate recovery, beneficial physiological adaptations take place, such as an improvement in exercise performance (14) and an enhanced immunosurveillance of some immune cell types (17). Nevertheless, if the practice of intensive exercise is repeated too frequently without maintaining an adequate balance with resting periods, the body may be unable to recover and adapt properly, resulting in transient performance declines and an exhaustion state called functional overreaching (OR) (14). This state may be reversed by adequate rest and recovery, however, if the overload of trainings takes place over weeks or months, it may lead to non-functional OR or it can even progress to the overtraining syndrome (OTS) (10,14,22,31,32). There is not full consensus on OR implications: some authors think it is a harmless reversible stage of the training process which is needed to improve performance (22,32), while other researchers state that its prevention is critical to benefit from exercise training (33). However, there is no doubt that OTS is a more serious undesirable condition that may involve health issues like fatigue, muscle damage, mental distress and higher rates of illness, among others, apart from the inability to perform at the expected levels (10,14,22,31,32). If not treated quickly and properly, the consequences of OTS may persist for months or even years (14,34).

The 60% of elite athletes experiences non-functional OR or OTS at least once in their career, whereas in amateur athletes this prevalence decreases to 33% (14). The high incidence of these conditions among professionals and the increasing participation of the general population in endurance events over the last decades has raised concerns regarding the impact of prolonged overly intense exercise on immune function and how to prevent it.

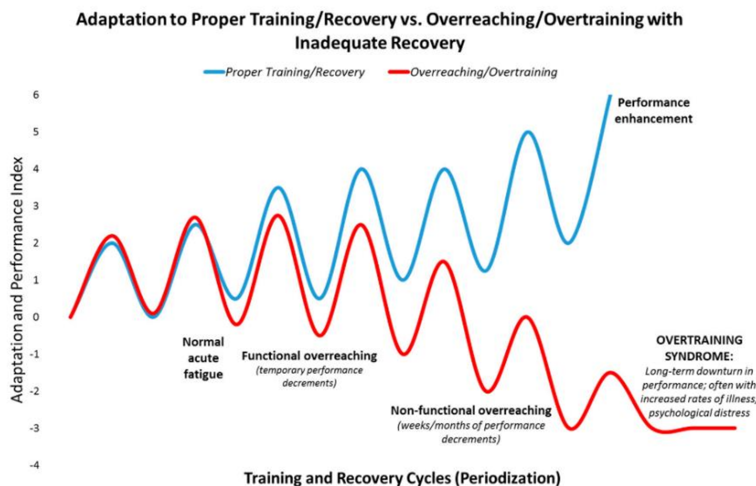

FIGURE 3. Comparison of the evolution of the exercise performance and the physiological adaptations in an adequate training/recovery balance with. Image from Nieman *et al.* (14).

Despite all the literature available, the isolated role of exercise in disrupting immunity and its open-window theory have been questioned lately (35,36). Increasing evidence suggests that many other uncontrolled factors, such as anxiety, psychological stress, sleep deprivation, travel and nutritional deficits prior to undertaking a bout of vigorous exercise, as well as the increased exposure to pathogens in a mass participation event, may be involved in the heightened incidence of infection observed in athletes.

1.2 EXERCISE AND CIRCULATING LEUKOCYTES

Blood leukocytes counts and the proportion of their subsets are normally quantified in exercise immunology studies, probably due to the easy access to this compartment and the existence of reference values. Despite all the variability between studies, it is well established that exercise is followed by an intensity- and duration-dependent leucocytosis, mainly due to the mobilization of neutrophils and lymphocytes from the marginal compartment. A single bout of moderate intensity exercise is able to induce a

two- to three-fold increase in leukocyte counts, whereas the practice of intensive exercise may increase them up to five-fold (21,30). High leukocyte counts are normally linked to infectious and inflammatory processes, however, the increase induced by exercise is a transitory effect (21). During the first hour after exercise cessation, the blood lymphocyte counts rapidly decrease until reaching a lymphopenia that may last up to 6 h, leaving the host more susceptible to infections and even opening a window for opportunistic pathogens. Parallel to this lymphopenia, the number of neutrophils keeps growing during the recovery period, reaching the peak a few hours after exercise cessation (25,37). The mechanisms responsible for these changes are multifactorial. As stated before, the demargination of leukocytes from the vascular endothelium occurs immediately in response to the exercise-induced changes in hemodynamics, such as an increase in cardiac output and in blood flow (21). Moreover, the overactivation of the sympathetic nervous system induced by exercise results in the release of catecholamines, which bind adrenergic receptors on leukocytes, mobilising lymphocytes, neutrophils, and monocytes in a lesser extent, from the endothelium but also from organs such as the liver, lungs and spleen into the blood compartment (21). The lymphopenia seems to be due to a redistribution of lymphocytes, mainly T helper (Th) cells, into non-lymphoid and lymphoid organs (38,39), as well as to an increase in apoptosis among highly differentiated T cells (40). B cells are similarly mobilized, although to a lesser extent because of their lower expression of adrenergic receptors (30). On the other hand, exercise also activates the hypothalamic–pituitary–adrenal (HPA) axis, causing the release of cortisol from the adrenal cortex, which stimulates the demargination of neutrophils from the endovascular lining, as well as the release of immature neutrophils from the bone marrow, explaining the second peak observed hours after exercise (21,41). Besides the demargination of neutrophils, the mentioned neutrophilia may also be associated with a delay in spontaneous apoptosis due to an exercise-induced increase in granulocyte colony-stimulating factor (G-CSF) production (42).

1.3 EXERCISE AND INNATE EFFECTOR CELLS

As mentioned before, innate immune system is the first barrier of defence against infection and unknown antigens. Unlike the adaptive response, it exists before the first contact with the antigen (Ag), thus being able to react immediately to potential pathogens. It comprises physical barriers (e.g., skin, mucus, cilia) that prevent the entry of pathogens into the human body. If such attempts fail, the innate effector cells (neutrophils, macrophages, and NK cells) get activated after the detection of pathogen-

associated molecular patterns (PAMPs) by the host's pattern recognition receptors (PRRs), which includes the toll-like receptors (TLRs), among others.

Overall, apart from the already mentioned leukocyte redistribution, exercise also alters the proportion and function of phagocytic cells and NK cells, and modulates the cell-surface TLRs expression, as well as the concentration of antimicrobial peptides and proteins in secretions, whose role is essential in the protection of mucosal surfaces. However, the impact of these changes on innate immune function depends on the intensity and duration of the exercise, as well as the basal fitness level and the existence of an adequate previous training program.

1.3.1 Granulocytes

Granulocytes are the major effector cells during the early phase of the innate immune response. There are three subtypes of granulocytes depending on their cell morphology and function: neutrophils, eosinophils and basophils. Neutrophils are the predominant subclass, and their main role is to identify, ingest and destroy microbial pathogens by phagocytosis and oxidative respiratory burst. Phagocytosis is an essential mechanism of the innate immune system for killing invading pathogens, or for preparing them for Ag presentation to T lymphocytes, leading to the activation of the adaptive immunity (43). Phagocytosis and the exposure to inflammatory mediators can lead to oxidative burst, which results in a dramatic increase in neutrophils oxidative metabolism and the release of reactive oxygen species (ROS) to combat certain pathogens (43).

Besides the exercise induced-neutrophilia mentioned previously, the function of neutrophils is also affected by exercise in an intensity- and duration- dependent manner (25). A single bout of moderate or intensive exercise is able to enhance unstimulated neutrophil degranulation and phagocytic and oxidative burst capacities (21,25,37); however, bacteria-stimulated degranulation seems to be inhibited (44). After a bout of exhausting exercise, such as completing a marathon, all these neutrophil functions appears to be inhibited (45–47). The enhanced phagocytosis after a moderate or intensive exercise has been associated with the release of glucocorticoids or catecholamines (44) and the upregulation of some surface receptors (48). Moreover, during the recovery period following exercise cessation, neutrophil oxidative burst continues to be enhanced after moderate or intensive exercise but remains impaired after exhaustive or overly prolonged exercise (21,47).

1.3.2 Monocytes and macrophages

Monocytes are the blood leukocytes with the highest size. They are also phagocytic cells and, upon infection or tissue damage, they rapidly migrate to the tissue, where they differentiate into macrophages, which play an essential role in the inflammatory response. Like neutrophils, monocytes phagocytic capacity increases after intensive (49) but not exhausting exercise (44).

Macrophages are a heterogeneous cell type able to polarize into M1 and M2 phenotypes, which are associated with pro-inflammatory and anti-inflammatory functions, respectively (50). Whereas M1 macrophages produce pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, IL-12 and IL-23, the M2 phenotype secretes anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β (50). Moderate intensity exercise seems to promote the phenotypic switching from M1 to M2 macrophages (51–53), potentially via activation of the nuclear transcription factor known as peroxisome proliferator activated receptor gamma (PPARγ) (53). Likewise, regular exercise reduces monocyte and macrophage infiltration into adipose tissue, probably due to the down-regulation of TLR4 expression (see section 1.4 for further details) (51) and the reduced release of monocyte chemotactic protein-1 (MCP-1) (54,55). Short-term moderate intensity exercise may also enhance macrophages adhesion and chemotactic abilities, their microbicide and phagocytic activities (21,48) and their capacity to produce nitric oxide (NO) (44).

In contrast, high-intensity and exhausting exercise may impair the function of macrophages. Preclinical studies report a decreased expression of major histocompatibility complex (MHC) class-II in peritoneal macrophages for several hours during recovery, leading to a lower Ag presentation ability (56,57). Moreover, intensive exercise can alter the ability of macrophages to produce ROS, which consist in oxygen-containing reactive molecules and free radicals produced by molecular oxygen reduction during normal cellular metabolism processes (58). ROS are involved in many physiological processes, such as gene transcription, cell signalling, apoptosis and immune function (58), as well as the regulation of macrophages survival (59), differentiation (60) and cytokine secretion (61). However, an excessive production and the accumulation of free radicals can impair the immune system functionality, leading to a systemic inflammatory status (62) that could contribute to the development of a number of pathologies (63), as well as muscle damage, physical fatigue, and an impaired exercise performance (64). Moderate intensity exercise increases ROS levels along with the body's antioxidant defences, contributing to the maintenance of a healthy oxidant status and enhancing the immune function (58). In contrast, intensive

exercise induces an overproduction of ROS, overtaking the antioxidant system's capacity and leading to oxidative stress (65). A lot of studies have shown excessive ROS production due to strenuous exercise training, both in athletes (66,67) and in preclinical studies (68,69). Nevertheless, Xiao *et al.* found that overload training on a treadmill for 4 (70) and 11 weeks (71) in mice inhibited the intracellular production of ROS in peritoneal macrophages.

1.3.3 NK CELLS

NK cells are a minor subset of lymphocytes that play an essential role in the innate immune response against virus-infected cells and transformed malignant cells. They act by directly killing these cells through the release of cytotoxic molecules, as well as triggering the adaptive immune response through cytokines secretion.

The impact of exercise on NK cells has been reviewed (30,72–74), concluding that there is an increase in circulating NK cell counts immediately after exercise cessation. However, in some studies, these numbers rapidly decrease, reporting values below basal levels 30 min later that can persist for more than 24 h. The imminent increase seems to be due to the down-regulation of adhesion molecules followed by a mobilization of prior attached cells induced by the release of catecholamines, since NK cells express a great number of adrenergic receptors on their surface (75). In humans, this mobilization preferentially affects the $CD56^{dim}$ NK cell subset, which is the predominant subset and has a strong cytolytic activity, rather than the $CD56^{bright}$ subset, which exerts less cytotoxicity but is an important producer of interferon (IFN)-γ (76,77). This fact could explain the increased cytotoxic activity after both moderate and endurance exercise reported in the majority of studies. Nevertheless there are some controversial results (30,72,73), probably due to differences among exercise protocols and intensities, as well as the physical condition of the participants. Moreover, the methodology used for assessing cytotoxicity and the way of expressing results seem crucial. Most studies assess cell cytotoxicity by coculturing effector cells (NK cells or a mix of lymphocytes) with a tumour target cell line. After incubation, the number of dead target cells represents the cytotoxic activity of these cells, although some authors adjust it by the number of NK cells, expressing it as individual/per cell cytotoxic activity, while others do not. On the one hand, when results are not normalized, the potential increase on NK cytotoxicity may merely reflect the exercise induced-increased proportion of NK cells among the effector cells mix (normally peripheral blood mononuclear cells, PBMCs, or lymphocytes isolated from a lymphoid tissue).

Nevertheless, there is no consensus even when cytotoxicity is normalized by the number of effector cells. Whereas some authors reported an increased cytotoxicity 2 h after intensive treadmill running (78), others found higher levels immediately after Qi-training, a type of martial art, which returned to basal levels after 2 h (79), no changes immediately after running up and down 150 stair-steps (77), or even an inhibited cytotoxicity after performing volleyball drills for 5 h/day, 6 days/week for 1 month (80). Bigley *et al.* found different changes in NK cytotoxicity after exercise when using K562 target cells, the most used cell line, with other tumour target cells of lymphoma and multiple myeloma origin (81). Specifically, they found no changes in individual cytotoxic activity when using K562 but an increase when using the other tumour cell lines.

The mechanism underlying the potential enhancement in NK cytotoxicity after exercise, besides the higher mobilization of the cytotoxic $CD56^{dim}$ NK cell subset (76,77), seems to involve the upregulation of the activation receptor NKG2C (81).

Overall, there is consensus about the mobilization of NK cells into the blood immediately after exercise cessation, although the duration of this increase may depend on the type, duration and intensity of exercise. In contrast, the available evidence about NK cytotoxic activity remains inconsistent, although a tendency could be stated in favour of increased cytotoxic activity after the practice of aerobic exercise (30,72–74).

1.4 TOLL-LIKE RECEPTORS

TLRs are a class of PRRs that are expressed by different subsets of immune and non-immune cells including monocytes, macrophages, dendritic cells, neutrophils, B cells, T cells, fibroblasts, endothelial cells, and epithelial cells of different tissues, such as the lungs, liver and skeletal muscle, among others (82). As mentioned before, TLRs are activated by the recognition of PAMPs or endogenous ligands such as damage-associated molecular pattern molecules (DAMP), which triggers the innate immune response through the NF-κB signalling pathway, resulting in the production of pro-inflammatory cytokines (82). TLRs also play an important role in the initiation and maintenance of the adaptive immune responses, especially on T cell activation, through the up-regulation of the expression of MHC-II and costimulatory molecules (CD80/86) on Ag presenting cells (83), as well as modulating the suppressor activity of regulatory T (Treg) cells (84). Since the down-regulation of TLRs on monocytes and macrophages seems one of the mechanisms by which moderate intensity exercise exerts anti-

inflammatory effects (85), the relationship between exercise and TLRs expression have been studied and reviewed by several authors (86–89).

TLR2 and TLR4 are the most studied TLRs, because they identify molecular patterns exhibited by numerous invasive pathogens and are the main TLRs involved in the pathogenesis of chronic low-grade inflammation (90,91). Malveira-Cavalcante *et al.* (88) focused on the roles of TLR2 and TLR4 in the inflammatory and anti-inflammatory effects of exercise according to the type of exercise and the frequency of training. They concluded that resistance exercise, both acute and chronic, does not seem to increase the expression of TLR2 and/or TLR4. In fact, the 75% of the studies about acute resistance exercise and the 67% of the chronic ones included in that review found a reduction of these TLRs in terms of mRNA or protein expression, whereas the rest did not find changes.

The results of the studies involving aerobic exercise protocols are more inconclusive. With regard to acute aerobic exercise, 40% of the revised studies reported a decrease, and 40% showed an increase in the TLR gene expression. For chronic aerobic exercise, 58% of the studies found a decrease whereas 25% observed an increase. In this case, the intensity of exercise appears to be a crucial factor. Most of the studies that found higher levels of TLR2 or TLR4 involved intensive exercise protocols, such as running for 50-60 min at 70% of maximal oxygen volume (VO_2max) (92,93) or running for 90 min at 16 m/min (94) in preclinical studies with rodents, and running 60 km as fast as possible (95) or performing 1 h of ergometer cycling followed by 1 h of treadmill running (96) in human studies. Nevertheless, another study found a higher gene expression of TLR2 in college students who played badminton for 2 h three times a week, which could be considered moderate intensity exercise (97). Hence, it seems that resistance exercise and moderate intensity aerobic exercise lower the expression of TLR2 and TLR4, which could be associated with decreases in inflammation, whereas intensive aerobic exercise may enhance their expression and potentially contribute to the inflammatory status and the immune alterations observed in athletes (25). However, further studies are needed to clarify the link between exercise intensity, these TLRs and inflammation.

Besides TLR2 and TLR4, changes in the expression of other TLRs due to exercise have also been reported by few authors. As TLR3 is implicated in the recognition of respiratory viruses, since it detects doubled stranded RNA, it could be an important factor in the potential risk of viral infections following intensive exercise. Perandini *et al.* found a lower TLR3 gene expression on circulating leukocytes after running for 30 min on a treadmill at 70% of VO_2max in adults with systemic lupus erythematosus (98). In line with this, Frellstedt *et al.* found down-regulation in mRNA levels of TLR3 in both

blood monocytes and pulmonary alveolar macrophages of horses after 8 weeks of intensive training (99). Likewise, Mignot *et al.* found that the gene response to lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid, which are TLR4 and TLR3 ligands, respectively, was inhibited in leukocytes isolated from bronchoalveolar lavage of horses following an exhaustion test on a treadmill (100). With regard to other TLRs, TLR7 mRNA levels were decreased in PBMCs after marathon running, although they were up-regulated compared to baseline the day after (101). After cycling for 1,5 h at 65% VO_2max in the heat (34 °C), TLR1 expression on monocytes was reduced, whereas TLR9 gene expression was not affected (102).

1.5 Systemic adaptive immunity

Adaptive immune responses are mainly carried out by lymphocytes that recirculate between blood and lymph going through various lymphoid and non-lymphoid organs to search for their cognate Ag. The impact of exercise on adaptive immunity depends on the intensity, duration and the type of exercise. Overall, exercise induces a mobilization of lymphocytes in the blood and modifies their function.

1.5.1 Lymphocyte redistribution

As previously stated, the practice of exercise induces an immediate lymphocytosis followed by a lymphopenia in the recovery period (25). The initial increase of circulating lymphocytes is the result of cell mobilization from the endothelium, the spleen or other lymphoid organs due to the downregulation of adhesion molecules mediated by adrenergic mechanisms.

Besides mobilization into the blood, lymphocyte redistribution also occurs between lymphoid and non-lymphoid organs. Spleen acts as a donor organ releasing lymphocytes during exercise, while the lung, bone marrow and Peyer's patches (PPs) serve as target organs (103,104). Krüger and Mooren (104) hypothesized that migration of lymphocytes into the lung might be an adaptation to increase ventilation and enhance the probability of contacting potentially harmful Ags, while the mobilization into the bone marrow may aim to stimulate the production of granulocytes and erythrocytes. These, together with the migration of lymphocytes into secondary lymphoid organs such as the PPs, may enhance immune surveillance.

Although all lymphocyte subsets participate in this redistribution, the magnitude of the mobilization depends on the number of adrenergic receptors expressed. The highest expression is found on the surface of NK cells, followed by CD8+ T cells, B cells and, last, CD4+ T cells (30), which explains the lower CD4/CD8 ratio found after exercise (105). The differentiation stage also affects the impact of redistribution, being highly differentiated T cells, even senescent T cells with limited antigenic specificity and reduced capacity for clonal expansion, the most mobilized (106). Simpson hypothesized that it could be a mechanism to eliminate senescent T cells by mobilizing them to the blood and expose them to proapoptopic stimulus, in order to allow a larger space for naïve T-cell repertoire (107). In line with this, an increased apoptotic of highly differentiated T cells have been observed after acute intensive exercise (40,108). Teixeira *et al.* supported the model proposed by Simpson after observing an increase in terminal effector and effector-memory T cells alongside a decrease in naïve T cells in blood of elite swimmers during the season (109).

Furthermore, minor lymphocyte subsets such as NKT cells (110,111) and Tγδ cells (109,112), which exert non-MHC restricted cytotoxicity, are also mobilized in response to exercise. Anane *et al.* reported that the mobilization of Tγδ cells was even greater than that of CD8+ T cells, although it was lower than that of NK cells (112). Thus, the existing evidence supports that cytotoxic lymphocytes are preferentially mobilized during exercise-induced stress.

Lastly, T regulatory cells (Tregs), which are an essential subset in the maintenance of immune homeostasis and tolerance, are also reported by mobilized following exercise (113–116). Whereas moderate intensity exercise increases the number and suppression function of Tregs in the lungs and lymph nodes in mice (115), intensive exercise may decrease them, as observed both in animal models (116) and marathon runners (114). This decrease may be due to the exercise-induced release of glucocorticoids, since dexamethasone (synthetic glucocorticoid) exposure for 24 h inhibits the in vitro FoxP3+ expression in PBMCs (117).

1.5.2 Lymphocyte function

Apart from the changes in the distribution of lymphocytes, exercise also modulates some of their functions such as their proliferative capacity, their ability to produce cytokines and Igs or their cytotoxicity (when applicable), among other functions. Again, the effect depends on the intensity, the duration and the type of exercise.

1.5.2.1 Lymphocyte proliferation

Naïve lymphocytes proliferate after encountering an Ag to differentiate into functional effector cells. In humans, PBMCs proliferation have been assessed and it has been observed a higher specific proliferative capacity following vaccination in active subjects than sedentary subjects (118).

However, the impact of moderate training in non-specific lymphocyte proliferation remains inconclusive, since a few studies reported a decrease (119) and others no changes (120–122). Intensive training, either acute (120,123) or chronic (124,125), appears to reduce lymphocyte proliferation capacity in response to mitogens. Siedlik *et al.* concluded that the magnitude of this inhibitory effect is proportional to the duration of exercise, whereas intensity has a lesser impact (123).

In preclinical studies, it has been assessed proliferative capacity in other lymphoid tissues. Surprisingly, most of the studies found a higher proliferative capacity of splenocytes (126,127) and mesenteric lymph nodes lymphocytes (128) after 6 (126,128) or 10 weeks (127) of high-intensity exercise training, such as swimming for at least 1h/day and, in some cases, with a load of 5.5-6% body weight attached (126,128). In contrast, Peijie *et al.* found a reduced splenocyte proliferation in response to stimulation with concanavalin A (ConA) or LPS in intensively trained rats, compared with both moderately trained and sedentary rats (129).

1.5.2.2 Cytokine secretion and Th1/Th2 balance

The production of cytokines by lymphocytes, mainly Th cells, is essential in the regulation of inflammation and the immune response against infection. Depending on the cytokines they secrete, Th cells can be classicaly differentiated into two main subgroups, type 1 (Th1) and type 2 (Th2) Th cells. Th1 cells promote cell-mediated immunity to fight intracellular pathogens, like viruses and intracellular bacteria, and are characterized by the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-2. On the other hand, Th2 cells produce anti-inflammatory cytokines like IL-4, IL-5, IL-10, IL-13 and IL-6. IL-6 has pleiotropic properties (both pro- and anti-inflammatory), promoting humoral immunity to eradicate extracellular pathogens. Under physiological circumstances, Th1 and Th2 cytokines are kept in a relative balance; nevertheless, chronic immune-mediated disorders, such as asthma, rheumatoid arthritis (130) or even cancer (131), have been associated with an imbalance of such ratio.

Moderate intensity exercise may contribute to maintain the Th1/Th2 balance (132,133), avoiding the age-related reduction of Th1 cells (134), or enhancing Th1 bias when there is a viral infection (135). On the contrary, high intensity exercise suppresses Th1 responses, disrupting the Th1/Th2 balance and increasing susceptibility to infection (30,132,136). This suppression can last for several hours or even days (132). For instance, a preclinical study reported the inhibition of Th1 response 7 days after exercise cessation in rats trained with a progressively increasing load for 9 weeks (137). This could be explained by the inhibition of IL-12 production induced by the release of catecholamines and glucocorticoids, interleukin that is the main promoter of the Th1 polarization, (138). In addition, Th1 cells are more susceptible to changes in catecholamines than Th2 cells because they express a higher number of β_2-adrenergic receptors (139). Other mechanisms may be involved, such as a reduced expression of different transcriptional factors, such as the signal transducer and activator of transcription 4 (STAT4), Th1-specific T box transcription factor (T-bet) or interferon regulatory factor-1 (IRF-1), among others, which are essential for the IFN-γ production by Th1 cells (140).

1.5.2.3 *Immunoglobulin production*

The main function of B cells is to differentiate into plasma cells upon Ag stimulation, which will lead to the production of Igs. Physical exercise induces changes in Igs concentrations, however, the effect depends on the intensity, duration and the type of exercise.

In general, moderate intensity exercise helps maintaining an optimal antibody (Ab) production against stressors (141) and ageing (142). In line with this, several studies reported a promising enhancement of the humoral immune response to vaccination following moderate exercise, both acute and chronic, in older adults (over 60 years of age) (142–146), where clinical vaccine efficacy is normally lowered. Nevertheless, the potential use of exercise as an adjuvant on younger subjects remains a bit inconclusive, since data are more limited and some studies reported improvements (147–149) and others no changes (150) or even different outcomes depending on the sex of the participants (151). In addition, it has been assessed the impact of exercising at the time of receiving whether the full-dose or a half-dose of a pneumococcal vaccine, reporting the immunoenhancing effect of exercise only in the subjects receiving the lower dose (152). Taken together, these data suggest that exercise may be a good adjuvant when vaccine immunogenicity is low or in cases where immune function is impaired.

Besides immune responses to vaccination, only a few authors have studied the impact of moderate intensity exercise on serum concentrations of Igs, finding contradictory results (153–155). For instance, Campbell *et al.* found an increased serum IgA concentration in postmenopausal women that performed 60 min of stretching once a week for 3 months, while the concentrations of IgG and IgM remained unchanged (153). However, Martins *et al.* reported higher levels of plasma IgA and IgM in older adults after performing moderate intensity aerobic exercise for 45 min, three times per week for 16 weeks (154). Furthermore, a systematic review and meta-analysis published recently by Chastin *et al.* concluded that no statistically significant effect of moderate exercise was detected for serum IgA, IgG or IgM (155).

Immunoglobulin production following intensive exercise seems to be either depressed (156–158) or unchanged (41), although some controversial results have been reported, especially with regard to IgG. For instance, McKune *et al.* found a reduced concentration of IgM and IgD in serum after running an ultra-marathon, whereas that of IgG was substantially increased (159). This rise in serum IgG concentration is in line with some preclinical studies (160,161) and may be associated with an increased IgG half-life (162) and a specific IgG N-glycosylation profile that may improve its affinity with the Fc receptor (163).

Lastly, most of the clinical studies assessing the impact of exercise on humoral immunity focus on changes in salivary IgA concentration, due to its essential role in mucosal immunity and the non-invasive sampling method (see section 1.6 for further details).

1.6 EXERCISE AND THE MUCOSA-ASSOCIATED LYMPHOID TISSUE

The mucosa-associated lymphoid tissue (MALT) is the largest immune component of the body and comprises half of the whole lymphocyte population, which, together with its constant exposure to Ags, makes it the starting point for a great number of immune responses. According to the mucosal site, the MALT can be divided into different types, among others, the salivary duct-associated lymphoid tissue (DALT) and the gut-associated lymphoid tissue (GALT), which defends the gastrointestinal tract against infections (164).

Immune responses developed in a particular MALT structure influence the immunity of the entire MALT. This is because activated immune cells can recirculate between the different MALT mucosa and glands (165). In turn, the GALT interacts with the intestinal epithelium (see section 1.7) and the microbiota (see section 1.8) to maintain gut

homeostasis (166) (**Figure 4**), which can be disrupted by prolonged overly intense exercise (167).

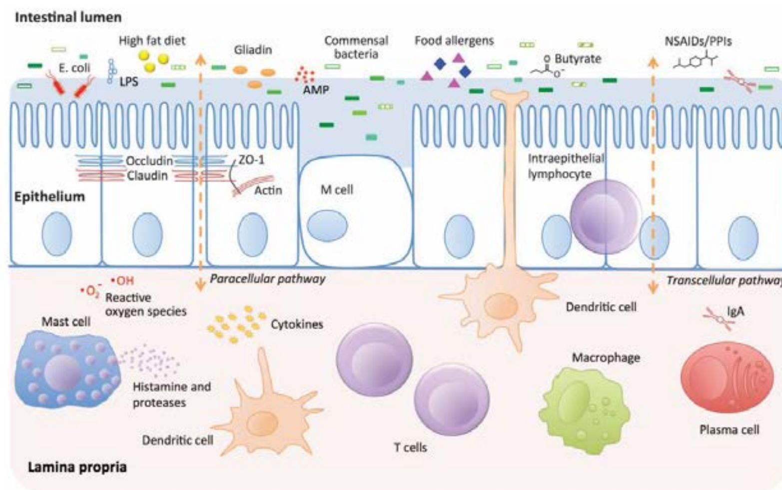

Figure 4. Structure of the intestinal epithelial barrier, by means of the mucus layer, the anti-microbial peptides and secretory IgA, the gut microbiota, the enterocytes bound by tight junction proteins and the immune cells from the gut-associated lymphoid tissue. Image from König *et al.* (166).

One of the main effector functions of the MALT is to produce and secrete IgA, which is the fundamental Ig isotype in the mucosal compartment. Here, it exerts many essential roles, such as Ag sampling, pathogens and toxins neutralization, as well as blocking pathogenic or excessive commensal bacteria (168,169).

The study of salivary IgA concentration in humans is one of the most used biomarkers to assess the effect of exercise on MALT function. Overall, it seems that regular sessions of moderate exercise increase salivary IgA concentration, whereas intense exercise may decrease it, explaining, at least in part, the higher susceptibility to mucosal infections observed in athletes (25,170,171). However, some authors have reported unchanged (172–175) or increased (176,177) levels of salivary IgA following intensive exercise. The mechanisms underlying the potential reduction in salivary IgA following prolonged intensive exercise are still unclear, but they may be related to changes in IgA synthesis, exocytosis or transcytosis due the prolonged overactivation of the sympathetic nervous system and elevated cortisol release (30). For instance, Kimura *et al.* found a down-regulated polymeric Ig receptor (pIgR) expression in salivary glands of rats submitted to an exhaustion test on a treadmill (178). Furthermore, it must be considered that changes on the GALT function may also play a role, since IgA-producing memory B cells

activation mainly takes place in GALT structures, like the PPs, isolated lymphoid follicles and the appendix, and then migrate to the salivary glands (165).

Besides IgA, other biomarkers of MALT dysfunction have been studied. On the one hand, salivary IgM play a less significant role than IgA in mucosal immunity, but in situations where IgA is deficient, its concentration usually increases as a compensatory mechanism (165). However, there is no consensus on the impact of prolonged intensive exercise on salivary IgM content, since some studies reported a parallel decrease with IgA levels, which could be explained by the potential down-regulation of pIgR (179), while others reported no changes (180), or even an increase (181). On the other hand, the innate salivary antimicrobial peptides α-amylase, lactoferrin and lysozyme, which, together with secretory IgA, confers the first line of defence against pathogens at mucosal surfaces, can also be modulated by intensive exercise (29,30). For instance, α-amylase production by the parotid gland seems to be increased as result of a strong sympathetic activation, as the one induced by prolonged intensive exercise, and several studies have reported an enhanced activity following exercise in an intensity-dependent manner (176,182). Increased secretion rates of lysozyme (29), lactoferrin (183) and salivary α-defensin (28,41) have also been reported.

1.7 EXERCISE, GASTROINTESTINAL ISCHEMIA AND INTESTINAL BARRIER FUNCTION

The immune cells of the GALT are constantly interacting with the intestinal epithelium, including enterocytes, tight junction (TJ) proteins, microbiota and the mucus layer, to establish and maintain an optimal intestinal barrier function, which allows the permeability of water, nutrients and electrolytes, while blocks the passage of pathogenic bacteria (166).

The gastrointestinal complaints observed in athletes (184–187) have commonly been associated with both a redistribution of blood flow and an increase in intestinal permeability (15,185,188,189). Intensive exercise induces an increase of blood flow to skeletal muscles and peripheral circulation, consequently reducing the blood supply to the gut and leading to intestinal ischaemia, which promotes cell damage and disrupts the epithelial barrier function (15,190). Even just 10 min of cycling at 70% VO_2max have been shown to induce a 20% decrease in portal blood flow of moderately trained men, whereas 1 h produced an 80% reduction (191).

The splanchnic hypoperfusion induced by intensive exercise correlates with a low intestinal damage and a higher intestinal permeability (192). The exercise-induced increase in gut permeability has been reported in numerous studies, and has normally been attributed to changes in the expression and phosphorylation status of the TJ proteins (193,194). TJs are specialized protein complexes that bind adjacent enterocytes on the lateral membranes, forming an extracellular barrier that regulates paracellular permeability (194). Claudins and occludins are tetraspan transmembrane proteins, whose extracellular components connect adjacent cells, whereas zonula-occludens (ZO) are intracellular regulatory proteins that link both claudins and occludins to the actin cytoskeleton, which upon activation shorten the epithelial cell, hence opening the TJs (194) (**Figure 5**). The contraction of the cytoskeleton depends on the phosphorylation state of the TJ proteins. Myosin light chain (MLC) kinase phosphorylates the MLC of the epithelial cytoskeleton, shortening and opening the TJ, whereas MLC phosphatase dephosphorylates the MLC, closing the TJ.

Hyperthermia (195), dehydration (188) and oxidative stress (194,196), which can be induced by intensive exercise, can disrupt the interaction between claudins, occludins and ZO, leading to the activation of phosphorylation enzymes tyrosine kinase, opening the TJs and increasing gut paracellular permeability. An increased TJ permeability may result in the translocation of harmful molecules, such as LPS, into the circulation, where they would interact with PAMPs and would induce the secretion of proinflammatory cytokines.

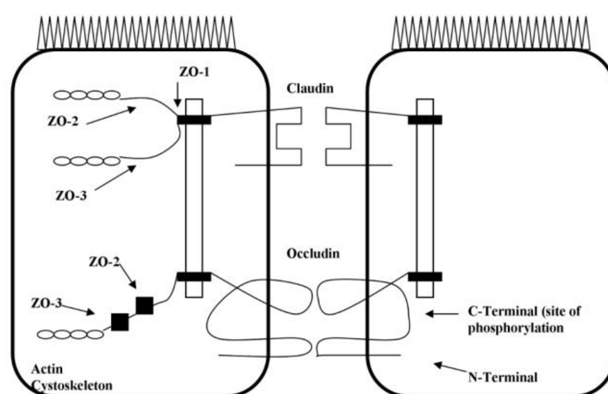

Figure 5. Structure of the tight junction barrier. Claudins and occluding are tetraspanning membrane proteins that interact with the intracellular regulatory proteins zonula-occludens (ZO). Image from Zuhl *et al.* (194).

TJ gene expression levels following intensive exercise in animals models have been determined by several authors (167,194,197–200). Whereas exercise modulates the

expression of the ZO proteins and occludin in a different manner depending on the intestinal segment assessed, claudin-2 seems to be upregulated in both the small intestine (duodenum, jejunum and ileum) and the colon (200). Due to its capacity to form cationic and water channels (201,202), high levels of claudin-2 expression have been associated with leaky-gut and the appearance of diarrhoea, which are GI complaints commonly observed in athletes (15,167).

Besides the study of TJ expression, there are a few studies assessing other permeability biomarkers, such as urinary sugar excretion (188,192,197,203) and the concentration of zonulin (193), calprotectin (192) or α1-antitrypsin (193) in faeces. Overall, gut permeability appears to increase following exercise in an intensity-dependent manner, although there are some controversial results probably due to methodological differences (15).

1.8 EXERCISE AND GUT MICROBIOTA

The gut microbiota plays an essential role in maintaining an optimal intestinal barrier function through competitive inhibition of pathogenic bacteria colonization and the production of short chain fatty acids (SCFA) with barrier-protective properties, like butyrate, among other mechanisms (166). Moreover, the interaction between the gut microbiota and the immune system, both innate and adaptive components, is crucial for keeping intestinal and extra-intestinal homeostasis and preventing immune-mediated diseases (204). There is increasing evidence indicating that gut microbiota composition and function can be modulated by exercise (205–208).

On the one hand, the regular practice of moderate intensity exercise, both in animals (209) and humans (210), seems to increase gut microbiota richness and diversity, which have been associated with better metabolic and immunological profiles. Some preclinical studies have reported an increase in the *Bacteroidetes/Firmicutes* ratio following moderate exercise (209,211), a pattern that has been associated with a lean phenotype, however, some clinical studies have found the opposite effect (210,212). Anyway, changes in this ratio should be interpreted with caution, since qualitative and quantitative changes in the abundance of the families, genus or species of these phyla may be more determinant. For instance, moderate exercise seems to increase the diversity among the *Firmicutes* phylum, including *Faecalibacterium prausnitzii* and other species from the genus *Oscillospira, Lachnospira*, and *Coprococcus*, promoting a healthier microbial environment (208). In this line, *Akkermansia muciniphila,* a mucin

degrader bacteria whose proportion negatively correlates with obesity and metabolic disorders, is more present in athletes than sedentary people (210,213). Preclinical studies have reported a positive association between aerobic capacity and *Lactobacillus* proportion (214–216), whereas an inverse relationship was found with the quantity of *Clostridium* (216).

With regard to the function of the gut microbiota, moderate exercise enhances the production of some SCFAs, such as acetate, butyrate or propionate (217–219), in part by modifying the expression of the acetate/butyrate coenzyme A (CoA) transferase and the propionate-regulating gene methylmalonyl-CoA decarboxylase (218), respectively. Moreover, moderate exercise seems to increase the abundance of butyrate producers bacteria, such as *Faecalibacterium* and *Rosebuira* (218,220), and that of some members of the genus *Veillonella,* which can convert exercise-induced lactate into propionate (221). This increase in SCFA concentration may explain some of the health benefits of moderate exercise, since these metabolites exert an essential role as metabolic regulators, improving insulin sensitivity and reducing inflammation, among other functions (222). Moreover, SCFAs mediate the metabolic cross-talk between the gut microbiota and skeletal muscle, thus, a higher production may improve energy metabolism and maximize substrate utilization (223,224), leading to increases in exercise performance.

On the other hand, some preclinical studies have found that strenuous exercise can decrease microbial richness and diversity and alter the composition of the gut microbial communities (225,226). Chaves *et al.* observed a decrease in the abundance of the *Lactobacillus* genus and an increase in that of the *Clostridium papyrosolvens* and *Clostridium ruminicantum* species in rats just after a single bout of high-intensity exercise (227). In this line, Batacan *et al.* also found higher levels of *Clostridium geopurificans* and *Clostridium saccharolyticum* in rats after performing high-intensity interval training (228), however, they also reported an increase in the abundance of *Lactobacillus johnsonii*, which disagrees with Chaves *et al.* (227) but agrees with other authors (214,216). A recent clinical study has also found gut dysbiosis in female endurance runners (229). For instance, they reported a higher abundance of *Haemophilus* and *Rothia* genera, which have previously been associated with gut inflammation, and in that of *Mucispirillum* and *Ruminococcus gnavus,* which are mucus-degrading bacteria (229). Overall, in contrast to moderate intensity exercise, strenuous exercise seems to decrease microbial richness and diversity and induce dysbiosis, however, evidence is still too limited to draw solid conclusions.

2. FLAVONOIDS AND IMMUNE SYSTEM

Flavonoids are secondary metabolites of plants that are regularly ingested in small quantities from many edible plants, constituting around 75% of the total polyphenol intake in Europe (230). The most consumed flavonoid subclasses in Europe are flavanols and flavanones (230). Flavanols are found in green tea, cocoa, grapes and other plant-based foods and beverages. They can be present in monomeric forms such as epicatechin, catechin, gallocatechin, epigallocatechin (EGC), and epigallocatechin gallate (EGCG), or in polymeric forms called proanthocyanidins or condensed tannins. Flavanones are exclusive to citrus fruits and include, mainly, hesperidin and naringin. Over the last decades, flavonoids have become a subject of increasing interest because of their numerous beneficial effects on human health, including as antioxidants (231), cardioprotectors (232) and their immunomodulatory (233) and anti-inflammatory (234) properties. In the athletic field, some of them have been recently proposed as ergogenic aids (235) and as a preventive strategy against intensive exercise-induced oxidative stress (11), inflammation (62) and immune disruption (14), which may result in a reduction of the increased incidence of URTIs and GI infections observed in athletes (236).

2.1 COCOA

Cocoa beans are the seeds of the cocoa tree (*Theobroma cacao L.*), a tropical plant indigenous to the rain forests of the equatorial regions of South-America. After fermentation, drying and subsequent processing, a paste called cocoa liquor is obtained, from which cocoa powder and chocolate will be made. Cocoa liquor contains cocoa solids and cocoa butter in almost equal proportions. The cocoa percent expressed in the current food packaging refers to the proportion of cocoa liquor. Cocoa powder is made by removing some of the butter from the liquor, whereas chocolate is made by combining the liquor with more butter and sugar (237).

Cocoa has been consumed since 600 B.C. by ancient civilisations and it was introduced in Europe in the 16th century. Cocoa has been traditionally used as a medicinal food, especially for treating angina and heart pain, although it has become a subject of increasing interest more recently because of the several beneficial effects on human health attributed to its flavonoids content (238).

2.1.1 Cocoa composition

Cocoa powder contains more than 300 chemical compounds. It provides about 26-40% of dietary fibre, 17% of it being soluble fibre and the 83% remaining insoluble, 15-20% of proteins, about 15% of carbohydrates and 10-24% of lipids. It is also a good source of vitamins (A, E, B and folic acid), minerals (Ca, Fe, K, Mg, P, Zn, among others) and bioactive compounds such as the methylxanthines theobromine and caffeine and a large number of flavonoids (239).

Cocoa is one of the richest foods in flavonoids. It is estimated that over 10 percent of cocoa powder dry weight is flavonoids (238), comprising about 58% of proanthocyanidins, 37% of catechins, and 4% of anthocyanidins (240). The proanthocyanidins contained are mainly dimers, trimers, or oligomers of flavan-3,4-diol, such as procyanidins B1, B2, B3, B4, B5, C1, and D (240). The predominant catechin monomer found in cocoa is (–)-epicatechin, with up to 35% of total flavonoid content. In smaller quantities, cocoa also contains (+)-catechin, (+)-gallocatechin and (–)-epigallocathechin, among others.

With regard to anthocyanidins, cyanidin-3-α-L-arabinoside and cyanidin-3-β-D-galactoside are the main ones present in cocoa powder (241). Nevertheless, the proportion of these compounds may vary during the manufacturing process of cocoa products. Moreover, there is some controversy surrounding the bioavailability of flavonoids. In the case of cocoa, the bioavailability of its flavonoids is considerated to be moderate (242). Monomeric and dimeric flavonoids are absorbed in the small intestine, whereas the oligo- and polymeric ones are able to arrive intact until the colon, where the colonic microbiota can metabolize them and generate secondary bioactive metabolites with even higher bioavailability and biological activities than the original flavonoids (243–245).

2.1.2 Antioxidant properties of cocoa

Cocoa has about a two-fold higher antioxidant capacity than red wine and green tea (240). All polyphenols have scavenging activity due to their chemical structure, which means they can directly neutralize free radicals (superoxide, hydroxyl, peroxynitrite, and nitric oxide, among others) by transferring their own electrons. They can also inhibit the enzymes involved in the production of free radicals (246). Several preclinical studies have reported the inhibitory activity of cocoa on the in vitro production of ROS by several kinds of cells (247–249). Cocoa has also shown to enhance the activity of the

endogenous antioxidant enzymes catalase and superoxide dismutase (SOD) in healthy rats (250), as well as to normalize these enzymatic activities in a rat model of oxidative stress (247). Clinical studies have found cocoa preventive effect on the formation of lipid oxidation products such as malondialdehyde (MDA) (251).

Moreover, (–)-epicatechin, among other polyphenols, has also demonstrated beneficial effects on mitochondrial biogenesis (252), which may contribute to the antioxidant potential of cocoa, since mitochondrial disfunction leads to an increased ROS production, among other undesirable effects. This flavonoid seems to stimulate the sirtuin 1 (SIRT1)-dependent signalling pathway, which results in a high expression of the peroxisome proliferator activated receptor c coactivator 1 (PGC-1) α (252), which is a transcriptional coactivator that regulates the expression of mitochondrial antioxidant genes (253). Moreover, it appears to regulate the activity of the transcription factors NF-κB and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (254), which are involved in the regulation of cellular resistance to oxidants.

A few studies have assessed the antioxidant potential of cocoa or its flavonoids on the exercise field. In exercised rodents, a dietary intervention with catechins increased glutathione reductase (GR) activity (255), while an intervention with cocoa fibre decreased lipid peroxidation (256). In the clinical area, Davison *et al.* (257) and Wiswedel *et al.* (258) reported that the acute intake of 248 mg and 186 mg, respectively, of cocoa flavonoids 2 h before exercise were able to increase total antioxidant status and prevent the increase in the lipid peroxidation markers F2-isoprostanone and MDA induced by exercise. This protective effect was also observed after 2-week interventions with dark chocolate (40 g/day, providing 98.7 mg of flavonoids) (259) and flavanol-containing milk chocolate (105 g/day, providing 168 mg of flavonoids). Taub *et al.* (260) carried out a longer study, involving 3 months of nutritional intervention with 20 g/day of dark chocolate, providing 205.8 mg of flavonoids, and found higher reduced glutathione levels in skeletal muscle biopsies of the treated group, which is one of the of the most important endogenous scavengers of ROS.

2.1.3 Anti-inflammatory properties of cocoa

Inflammation is the local response of tissues to an aggression caused by wounding or noxious agents, such as pathogens or chemicals, in order to protect the host and heal damaged tissue. The inflammatory response involves the migration of leukocytes from

the circulation to the site of tissue damage and the release of growth factors, cytokines, ROS and nitric oxide (NO), among other mediators. Several in vitro studies have shown cocoa ability to reduce the release of these mediators, although the in vivo evidence is still low.

Most of the studies assessing the anti-inflammatory properties of cocoa have focused on changes in the release of proinflammatory cytokines, although results remain quite controversial, probably due to the use of different cocoa derived products, such as cocoa powder, chocolate or different flavanol extracts. An in vitro study carried out by Dugo *et al.* (261) reported an inhibited secretion of TNF-α, IL-6, IL-1β, and IL-12 by proinflammatory M1 macrophages, while that of IL-10 was enhanced, suggesting a shift toward M2 macrophage polarization following a treatment with a cocoa polyphenol extract (261). Ramiro *et al.* (262) found similar results at the transcriptional level, reporting also a dose-dependent reduction in MCP-1. In a lymphoid cell line, stimulation with a cocoa extract also inhibited the secretion of IL-2 and downregulated the expression of IL-2 receptor α (CD25) on activated cells (263). In contrast, in vitro IL-4 release, the most representative Th2 cytokine, seems to be increased in cocoa-treated cells (263,264). A few authors have assessed the effect of the different flavanols present in cocoa, according to the length of their chain, on the release of cytokines, finding interesting results. In PBMCs, short chain flavanols (monomers, mainly (-)-epicatechin, and dimers) inhibit the release of TNF-α (265) and IL-1β (266) whereas long-chain procyanidins (hexamers to decamers) increase it, as well as that of IL-6 and IL-10 (267). With regard to IL-5, a cytokine involved in eosinophil maturation and the differentiation of B cells into IgA-producing plasma cells, the same authors reported an increased secretion following stimulation with monomeric and dimeric cocoa flavanols, whereas the larger procyanidin fractions inhibited it (268). Lastly, concerning TGF-β, an anti-inflammatory cytokine involved in tissue repair and regeneration, it has been reported a homeostatic effect of all the tested fractions, since they induced a higher secretion in low producers and a lower one in high producers (269).

In preclinical studies, the consumption of a diet containing 10% cocoa for two weeks or longer periods decreased the TNF-α release by peritoneal macrophages and the serum concentration of MCP-1 (270), whereas the levels of IFN-γ in serum and lymphoid tissues remained unchanged (271) and those of IL-4 have been reported to be either increased (272), reduced (271,273,274) or unchanged (275). In clinical studies, dark chocolate consumption increased mRNA expression of IL-10 in healthy men (276) and decreased the serum concentrations of TNF-α, IL-6 and MCP-1 in obese adolescent

boys (277). However, other authors have reported no changes in IL-6 (278), or even an increased release of TNF-α and IL-1β after a nutritional intervention with cocoa (279).

In the athletic field, no changes were found in plasma IL-6, IL-10 and IL-1ra concentrations after cocoa supplementation (257,259). These changes on the cytokine pattern may be associated with the inhibition of the NFκB-dependent transcription pathway induced by cocoa consumption (280), since this pathway is involved in the regulation of genes encoding cytokines and adhesion molecules, among others. In line with this, the serum concentration of intercellular adhesion molecule 1 (ICAM-1) and E-selectin was reduced 6 h after consuming 40 g of cocoa powder (280). Besides proinflammatory cytokines, the impact of cocoa on other inflammatory mediators has also been assessed. For instance, cocoa flavonoids, mainly (-)-epicathechin and small procyanidins (dimers to pentamers), have shown to inhibit the activity of some lipoxygenases, avoiding the conversion of arachidonic acid into leukotrienes, among other inflammatory mediators (281). Furthermore, di Giuseppe *et al.* (282) reported an inverse relation between dark chocolate consumption and serum C-reactive protein (CRP), a nonspecific marker of inflammation, in a healthy Italian population.

Flavonoids can also modulate intestinal inflammation. In Caco-2 cells, cocoa flavonoids promoted the synthesis of prostaglandin E_2 by activating cyclooxygenase 1, which has a strong protective effect on mucosal integrity (283). Using in vivo rodent models of ulcerative colitis, the administration of cocoa or its derived flavonoids attenuated the dextran sodium sulphate-induced colitis by inhibiting the phosphorylation of the signal transducers and activators of transcription (STAT)-1 and STAT-3 in colon cells (284), as well as the derived oxidative and inflammatory response (285).

2.1.4 Cocoa as immunomodulator

Clinical studies assessing the health benefits of cocoa have mainly focused on oxidative stress prevention and cardiovascular health. The immunomodulating properties of cocoa have been suggested in several human studies based on the mentioned anti-inflammatory properties. In the last few years, in vivo animal models have allow to elucidate the impact of cocoa on lymphoid tissue composition and lymphocyte function.

2.1.4.1 *Effects on lymphocyte composition and function*

Primary lymphoid organs comprise the bone marrow, in which leukocytes are produced, and the thymus, where T-lymphocytes mature. Preclinical studies have reported changes in lymphocyte composition of primary lymphoid organs after a dietary intervention with cocoa. Cocoa promotes the maturation of T cells in the thymus, accelerating the progress from immature double positive thymocytes, expressing low levels of TCRαβ, toward mature single positive cells, expressing high levels of TCRαβ (250,286).

Secondary lymphoid organs include spleen and lymph nodes. In the spleen, results vary among studies. Ramiro-Puig *et al.* reported a rise in the B cell proportion and a decrease in that of Th cells after 3 weeks of nutritional intervention with a 10% cocoa-enriched diet (C10 diet) (271), while Camps-Bossacoma *et al.* (286) found no changes in B cells, a higher Th cell proportion and a lower percentage of Tγδ, NK, Tc and NKT cells after 8 days of intervention with the same diet. In contrast, in the GALT, there is consensus about a decreased Th proportion in the mesenteric lymph nodes (MLN) following a dietary intervention with cocoa (273,275,286). Moreover, cocoa consumption for more than 3 weeks induced an increase in the proportion of NK, Tγδ and Tc cells in these lymph nodes (273,275). A longer study, which involved 4 weeks of nutritional intervention, also found a lower proportion Tαβ cells, due to the substantial decrease of Th cell percentage, and an increase in that of B cells (275). Furthermore, cocoa also modulates the lymphocyte composition of PPs, which are lymphoid aggregates found throughout the wall of the ileum region that, together with the MLNs, belong to the organized or inductor GALT. In particular, cocoa intake for 3 and 4 weeks decreased the Tαβ cell proportion, mainly due to a reduction in that of the Th subset, while increased the percentage of B, Tγδ and NKT cells in PPs (273,287), similar to the changes observed in in the MLNs. Moreover, in rats fed the C10 diet, PPs had higher proportions of CD25+, CD103+ and CD62L− cells, suggesting a higher cell activation and a mobilization of effector cells to the intestinal compartment (287). Lastly, cocoa's influence on the diffuse or effector GALT has also been assessed (287). Four weeks of nutritional intervention with cocoa also modulated the composition of intraepithelial lymphocytes (IELs), whereas no changes were observed in lamina propria lymphocytes (LPLs), both part of the diffuse or effector GALT. There was a higher percentage of Tγδ cells, both CD8αα and CD8αβ subsets, in IELs from the small intestine of rats fed cocoa-enriched diet (287).

Besides these phenotypic changes, cocoa consumption can also modulate lymphocyte function. Preclinical studies have shown cocoa's ability to modulate the proliferative

capacity of lymphocytes. In a rat model of colon cancer, a cocoa diet exhibited antiproliferative effects, which resulted in a lower number of pre-neoplastic lesions (288). However, in healthy rats, no changes in MLN and spleen lymphocytes proliferation were observed after a nutritional intervention with the C10 diet (273,274). In humans, an increase in NK cell activity in response to influenza vaccine after consuming a cocoa beverage for 3 weeks has been reported (289).

2.1.4.2 *Effects on humoral immune response*

Besides altering the lymphocyte composition of systemic and intestinal lymphoid tissues, cocoa also induced functional changes in these cells. Despite the increase in B cell proportion observed in some compartments (271,273,275,287), the Ab production has been found to be attenuated after cocoa consumption. In particular, the intake of a C10 diet for 3 weeks was able to decrease serum IgG, IgM and IgG concentrations in 3- and 6-week-old rats (271,286). However, other studies reported no changes in these Igs after 2 weeks of dietary intervention with cocoa flavonoids (290), or even different effects depending on the rat strain used (291). With regard to IgG isotypes, a C10 diet for 3 weeks resulted in lower IgG2b levels but higher IgG2a levels in 3-week-old rats (292). Nevertheless, when the dietary intervention started later, at 6 weeks of age, a decrease in serum IgG2a concentration and an increase in that of IgG2c were found in cocoa fed rats (293). Then, although cocoa seems to inhibit systemic Ab synthesis in preclinical studies, the effect depends on the Ig isotype, the animal strain and age, as well as the length of the nutritional intervention.

The attenuating effect of cocoa on Ig synthesis may be interesting for the nutraceutical management of immune-mediated diseases that involve the formation of antibodies with a pathological role, such as allergy or autoimmune diseases. In this line, preclinical studies have also shown cocoa's ability to reduce the synthesis of specific Igs during an immunization with ovalbumin (274). The most inhibited Ig isotypes were specific IgM, IgG1, IgG2a and IgG2c, whereas IgG2b levels were increased following a C10 diet (274). In rats, IgG2b is associated with a Th1 immune response, whereas IgG1 and IgG2a are associated with a Th2. Therefore, these preclinical results suggest a lowering effect of cocoa on Th2 response. This could be due to cocoa flavonoids, since other flavonoids, such as genistein (294), chrysin and apigenin (295), have shown similar effects. In food allergy models, cocoa is able to lower the synthesis of specific IgE by 60-70% (272,296). In these studies, the influence of cocoa on allergic manifestations was not assessed, however, in similar studies evaluating the impact of other flavonoids, such as quercetin

(297,298) and myricetin (299), the attenuating effect on IgE was directly associated with lower allergy symptomatology. Lastly, this attenuating effect of cocoa on specific humoral immunity has also been reported in experimental models of autoimmune arthritis (270) and atopic dermatitis (300).

In the mucosal compartment, this effect is even more evident. On the one hand, the consumption of a diet containing 4% cocoa for 2 weeks was able to decrease the faecal concentration of IgA (273). When the cocoa content in the diet increased up to 5 or 10%, this reduction could remain longer (290–293), at least, 7 weeks after the start of the nutritional intervention (292). In contrast, a 2% cocoa diet did not modify faecal IgA, confirming a dose-dependent effect (293). On the other hand, lower IgA and IgM levels have been found in other mucosal compartments, such as submaxillary and parotid salivary glands, MLNs, PPs and small intestine wash (273,275,291–293,301).

Theobromine seems to be the main responsible for the effects of cocoa on both systemic and mucosal Abs (286). Camps-Bossacoma *et al.* (286) found a similar decrease in the concentration of IgG, IgM and IgA in serum and that of IgA in faeces in rats fed C10 diet and rats fed 0.25% theobromine diet, which corresponds to the amount provided by the C10 diet. In contrast, Massot-Cladera *et al.* (301) observed different effects on humoral immunity after a C10 diet and a 5% cocoa fibre (CF) diet (same amount provided by the C10). Whereas the C10 diet decreased the concentration of IgA and IgM in PPs and MLN, the CF diet increased them. However, both C10 and CF diets reduced the IgA content in small intestine wash and salivary glands. The intake of the CF diet also induced a higher IgA concentration in serum. In order to elucidate the molecular mechanisms involved in these changes, further analysis of the gene expression of related molecules were carried out (292,301) (**Figure 6**). Pérez-Berezo *et al.* (292) focused on the intestinal compartment and observed that after 7 weeks with the C10 diet the direct interaction between T and B cells by CD40 was not affected by the diet in the PPs and MLNs. However, cocoa diet decreased the gene expression of IL-6. As IL-6 plays an important role in the differentiation and maturation of B cells into IgA+ B cells, these results suggested a lower generation of IgA+ B cells and/or the production of B cells with a lower ability to synthetize IgA. Moreover, the dietary intervention also lowered the number of IgA+ B cells reaching the intestinal lamina propria (287) by down-regulating the expression of chemokines and chemokine receptors involved in gut homing, such as C-C chemokine ligand (CCL)-28 and C-C chemokine receptor (CCR)-9, respectively, mediated in part by the down-regulation of retinoic acid receptors (RAR). Camps-Bossacoma *et al.* (287) also found a reduced TGF-β1 gene expression in small intestine of rats fed C10 diet for 4 weeks,

which suggested a lower IgA switching. Overall, cocoa modifies IgA+ B cell differentiation, IgA synthesis and B cell homing. In contrast, it does not seem to affect IgA transcytosis, since pIgR gene expression was unchanged in rats fed C10 diet for 3 (293,301) or 7 weeks (292).

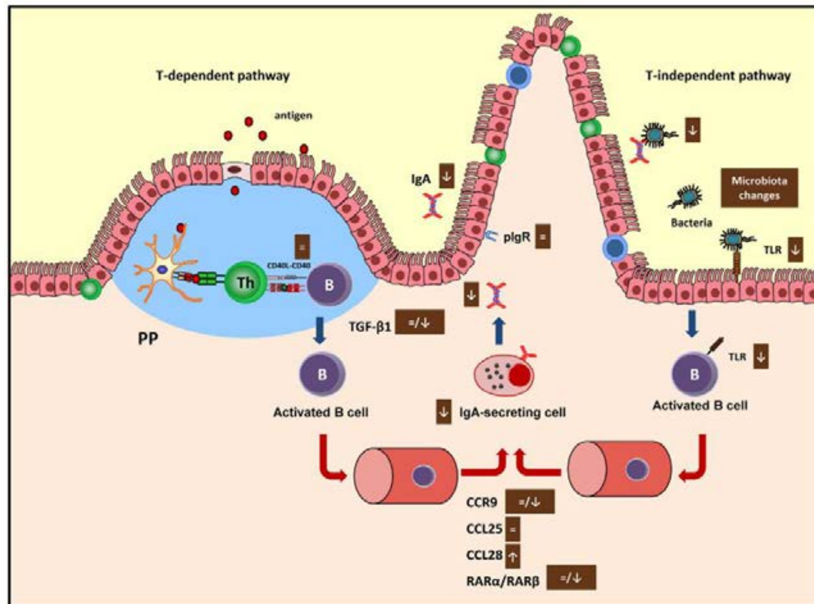

Figure 6. Summary of the mechanisms involved in the down-regulation of intestinal IgA induced by cocoa. Arrows indicate increases or decreases, equals signs mean no changes. Ig, immunoglobulin; PP, Peyer's patches; pIgR, polymeric immunoglobulin receptor; TLR, toll-like receptor; CCR, C–C chemokine receptor.

Massot-Cladera *et al.* (301) evaluated the influence of the C10 and CF diets on the gene expression of some of these molecules in salivary glands. The C10 diet, but not the CF, induced a drastic decrease in the IgA gene expression and an increase in that of CCL28, probably as a compensatory mechanism, without altering the gene expression of TGF-β1, RARs and pIgR. In contrast, the CF diet did not affect the IgA gene expression, but it induced a reduction in that of TGF-β1, RARα and pIgR. These results suggested that the changes on Ig production induced by the C10 and the CF diet may involve different mechanisms. Whereas cocoa appears to modify IgA synthesis and B cell homing, the CF diet may alter the homing and transcytosis of IgA.

On the other hand, IgA+ B cells can also be generated in a T-cell independent manner that involves TLR signalling, among other elements. Flavonoids are able to modulate TLR-mediated signalling pathways (302). In particular, a lower proportion of TLR4+ cells in IELs (287) and a reduced TLR4 gene expression in small intestine (292) have been

observed after a nutritional intervention with cocoa. This also contributes to the down-regulating effect of cocoa on IgA, since the activation of TLR4 signalling increases B cell recruitment to the lamina propria, which may result in a higher secretion of IgA (303). Lastly, a microarray analysis revealed a drastic down-regulation of the gene tachykinin 4, which has been described as a promoter of B lineage cells (304). Taken together, all these results confirm that the inhibitory effect of cocoa on humoral immunity is multifactorial, and that cocoa modulates immune function at multiple sites and by different components.

2.1.4.3 *GUT MICROBIOTA*

The relationship between cocoa and gut microbiota is bidirectional. As previously stated, cocoa procyanidins reach the colon intact, where they are metabolized by the intestinal microbiota. The resulting colon bacterial metabolites include bioactive compounds with higher biological activities that can be better absorbed into the bloodstream than the original flavonoids (243–245). In addition, cocoa flavonoids, and the generated bacterial metabolites, can modulate microbiota composition and function.

An in vitro study using a batch-culture model reported some beneficial changes on gut microbial composition after incubation with cocoa flavanols (305). In particular, an increased growth rate of *Bifidobacterium* spp., *Lactobacillus* spp. and *Clostridium coccoides-Eubacterium rectale* was observed, whereas that of *Clostridium histolyticum* was reduced. Animal studies reported similar effects: the consumption of diets enriched in cocoa or cocoa flavonoids considerably decreased the faecal proportion of *Clostridium, Bacteroides* and *Staphylococcus* genus in rats (291,306). In pigs, an increased growth of *Lactobacillus* and *Bifidobacterium* spp. was observed after supplementation with cocoa powder for 27 days (307).

In a diabetes rat model, the intake of a cocoa diet restored the diabetes-induced changes on microbial composition and increased the proportion of acetate-producing bacteria, especially *Blautia,* increasing the faecal concentration of acetate accordingly (308). Massot-Cladera *et al* (309) also evaluated the production of caecal SCFA in rats fed C10 diet, finding a higher concentration of faecal butyrate than in control animals. Furthermore, the influence of other bioactive compounds besides polyphenols have also been studied in rats. Cocoa fibre consumption (CF diet) for 3 weeks increased *Bifidobacterium* and *Lactobacillus* spp. counts and induced a higher total SCFA concentration, mainly due to increases in acetate, butyrate and propionate (309). The

intake of a theobromine enriched diet for 15 days, but not the C10 diet, lowered the counts of *Bifidobacterium* and *Streptococcus* spp. and *Clostridium histolyticum-C. perfingens* group, and, like the C10 and CF diets, induced an increase in the butyrate production (310). Furthermore, some of these studies showed changes in the proportion of commensal bacteria bound to IgA (IgA-CB) after the intake of experimental diets based on cocoa or any of its bioactive components (309,310). Whereas diets rich in cocoa or theobromine decreased the proportion of IgA-CB (310), a diet rich in cocoa fibre increased it by more than threefold (309). IgA can bind bacteria for different reasons, such as neutralizing pathogenic bacteria or transporting commensal bacteria to the intestinal epithelium (169), thus, further analysis may be needed to clarify the impact of these changes. Nevertheless, IgA appears to have a higher affinity to colitogenic bacteria (311).

Human trials evaluating the influence of cocoa on microbiota are scarce. Tzounis *et al.* (312) also reported an increase in the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. in healthy adults after consuming a cocoa flavanol-enriched beverage for 4 weeks, suggesting that cocoa flavonoids may behave as prebiotics also in humans, as described previously in rats (309). Further studies are needed to confirm this prebiotic potential, as well as to clarify the optimal cocoa processing and food matrix, since these factors seem to determine the kind of effects induced on microbiota composition and function (313,314).

2.2 HESPERIDIN

Hesperidin is a flavanone mostly found in citrus fruits. In particular, it is the most predominant flavonoid found in sweet oranges (*Citrus sinensis*). The higher levels of hesperidin are found in citrus peel. However, orange juice can contain between 300-600 mg/L of hesperidin, because sometimes peel is incorporated during the industrial processing (315). This means that the consumption of a glass containing 200 mL of orange juice can provide up to 120 mg of this flavanone.

Hesperidin has a chiral carbon that generates two isomeric forms: 2S- and 2R-hesperidin, being the 2S the most predominant in citrus fruits (about 92% of the hesperidin content in orange juice) (316). Moreover, 2S-hesperidin has demonstrated a higher bioavailability than 2R-hesperidin (317). Similarly as cocoa polyphenols, both enantiomers of hesperidin are metabolized by the intestinal microbiota, mainly in the proximal colon, which results in the formation of smaller phenolic compounds like the

aglycone hesperetin that exerts bioactive activities and has a higher bioavailability than hesperidin per se (318–320). Due to its increased absorption, a lot of interventional studies have directly assessed the effects of hesperetin. In recent years, hesperidin and hesperetin have become a subject of increasing interest because of their numerous health promoting effects (321), mainly due to their antioxidant and anti-inflammatory properties (322).

2.2.1 Antioxidant properties of hesperidin

The antioxidant properties of hesperidin are not only limited to the free radical scavenger activity conferred by polyphenols chemical structure, but also to other mechanisms. This flavanone has also shown to reduce oxidative stress through the activation of the Nrf2 signalling pathway (323), as reported for epicatechin (254) and resveratrol (324). This effect seems to be dose-dependent and results in a reduced ROS production as observed in cisplatin-treated cells (323). Furthermore, hesperidin can inhibit lipid peroxidation, as indicated by reduced levels of myeloperoxidase and MDA in animal models of colitis (325), hepatic injury (326) and cisplatin-induced oxidative stress (323). Lastly, hesperidin has shown to enhance the endogenous scavenger antioxidant systems in both in vitro and in vivo studies. The ex vivo treatment with hesperidin resulted in enhanced levels of SOD and glutathione in kidney cells of cisplatin-treated mice (323). In aged rats, hesperidin supplementation prevented the reduction of catalase, SOD and GR activities in the liver induced by aging (327). Similar effects were observed in a rat model of pleurisy (328).

The antioxidant properties of hesperidin have also been reported in the exercise context. De Oliveira *et al*. (329) observed an enhanced antioxidant capacity and a reduced lipid peroxidation in hesperidin-supplemented rats submitted to interval swimming. Likewise, Estruel-Amades *et al.* (69) reported the effect of hesperidin supplementation preventing the increase in ROS production and the decrease in SOD and catalase activities in thymus and spleen induced by exhausting running exercise in rats. Similarly, hesperetin supplementation for 6 weeks increased the reduced/oxidized glutathione ratio in runner aged mice (330). Furthermore, the antioxidant effects of hesperidin resulted in an improved exercise performance (69,330). A recent human trial has also demonstrated the enhancing effect of hesperidin supplementation on antioxidant capacity (331). In particular, the intake of 2S-hesperidin for 8 weeks resulted in higher SOD capacity and lower oxidized glutathione levels. In addition, these changes were accompanied by a better exercise performance (332).

2.2.2 Anti-inflammatory effects of hesperidin

In vitro and in vivo studies have reported the anti-inflammatory properties of hesperidin and its aglycone, hesperetin. Comalada *et al.* (333) reported an inhibitory effect of hesperetin on in vitro TNF-α production by LPS-stimulated macrophages. In cultured human synovial cells, hesperetin also reduced the production of proinflammatory mediators, such as IL-6 and matrix metalloproteinase-3 in response to IL-1β stimulation (334).

With regard to the expression of adhesion molecules, hesperetin avoided the increase in vascular cell adhesion molecule 1 induced by TNF-α stimulation in endothelial cells (335,336). Moreover, hesperidin lowered the expression of ICAM-1 in endothelial cells cultured with high concentration of glucose (337). These changes in adhesion molecules may result in a decreased monocyte adhesion to endothelial cells, which would suggest a potential protective effect of hesperidin and hesperetin on cardiovascular health.

In healthy rats, hesperidin supplementation for 4 weeks did not modify the production of IFN-γ, MCP-1, IL-4, IL-10, or TNF-α by anti-CD3/CD28-stimulated MLN lymphocytes, although it reduced the IFN-γ and MCP-1 concentrations in small intestine wash (338). Macrophages isolated from hesperidin-supplemented mice produced higher levels of IL-12 than control mice (339). However, when these macrophages were stimulated with LPS ex vivo, hesperidin-supplemented mice had the opposite effect, decreasing IL-12 production, together with a decrease in IL-10 and TNF-α secretions. These results suggest the immunomodulating effects of hesperidin, but the influence of this flavanone may be different depending on the basal immune status. Mice supplemented with hesperidin prior to undergoing irradiation had lower serum concentrations of IL-1β, IL-6 and TNF-α than control mice (340). Moreover, in rodent models of Th2-mediated diseases, hesperidin supplementation inhibited the IL-4 production in splenocytes and that of IL-5 in the bronchoalveolar fluid (341), while it increased the secretion of IFN-γ by MLN lymphocytes (342), suggesting an attenuating effect on Th2 immunity.

In human trials, supplementation with 500 mg of hesperidin daily for 3 weeks reduced the plasma concentration of CRP, soluble E-selectin and serum amyloid A in plasma in patients with metabolic syndrome (336). In patients with type-2 diabetes, hesperidin supplementation for 6 weeks resulted in lower plasma concentrations of CRP and IL-6 (343). These data suggest the potential therapeutic role of hesperidin in the chronic inflammation involved in metabolic diseases.

A clinical nutrigenomic study has also demonstrated that the consumption of hesperidin for 4 weeks modulates blood leukocyte gene expression in healthy volunteers, affecting specially those genes associated with anti-inflammatory and anti-atherogenic activities (344). In particular, hesperidin down-regulated the expression of genes encoding chemokines, such as MCP-1, CCL26, CX3CR1 and CXCL17, and the cytokine IL-4, among other genes involved in the processes of inflammation. All these changes may be explained by the up-regulation of NF-κB inhibitor gene expression found after hesperidin consumption, which may result in a reduced activation of NF-κB and, consequently, in a decreased secretion of chemokines and cytokines. Moreover, they compared the gene expression profile obtained in these subjects with the one obtained after an intervention with orange juice and concluded that at least the 50% of the modifications induced by orange juice consumption seemed to be due to hesperidin.

2.2.3 Hesperidin as immunomodulator

Finally, there are few in vivo studies assessing the influence of hesperidin on immune cells and lymphoid tissues. The oral administration of 100 or 200 mg/kg of hesperidin for 4 weeks to rats increased the proportion of Tαβ cells and reduced that of B cells in MLN of both healthy (338) and immunized animals (342). The proportion of both Tαβ subsets, Th and Tc, was also increased in the spleen of mice supplemented with hesperidin for 6 weeks (340). When hesperidin was given through the diet (0.5% hesperidin diet), there were no changes in the composition of MLN lymphocytes. Nevertheless, rats fed hesperidin diet had a higher proportion of Tγδ cells in IELs, while the percentage of these cells was decreased in LPLs. In addition, in LPLs from rats following an hesperidin diet, the proportion of B and Th cells was higher, while the percentage of the cytotoxic cells Tc and NKT cells was lower (342).

The effect of hesperidin on lymphocyte function was also assessed in some studies. Lymphocyte proliferation was unchanged in MLN of healthy rats (338) but increased in spleen of both healthy rats (345) and irradiated mice (340). The cytotoxic activity of NK and Tc cells was increased after stimulation with hesperidin (345). Unlike cocoa flavonoids, hesperidin seems to increase intestinal IgA, although no changes have been observed in IgA serum concentration (338,342). In ovalbumin sensitized rats, hesperidin supplementation did not modify the production of serum specific antibodies (342).

With regards to the gut microbiota, hesperidin has also shown a potential prebiotic effect. In healthy rats, an increased in *Lactobacillus* spp. abundance after 4 weeks of hesperidin supplementation has been reported (338). Moreover, hesperidin resulted in a higher proportion of IgA-CB. Nevertheless, Unno *et al.* (346) found no effects on microbial composition when administering the hesperidin through the diet (1% hesperidin diet). Surprisingly, they did find effects when adding to the diet the aglycone hesperetin (0.5% hesperetin diet). In particular, they found a reduced faecal proportion of *Clostridium* subclaster and an increased caecal concentration of SCFA after 3 weeks of dietary intervention with hesperetin. They attributed the controversial results to the higher inhibitory effect of hesperitin on starch digestion.

A clinical study has also reported prebiotic effects after 2 months of intervention with a commercial pasteurized orange juice (347). Particularly, increases in the population of faecal *Bifidobacterium* spp. and *Lactobacillus* spp. were found in healthy women after the orange juice intervention, which was accompanied by a higher production of total SCFAs, mainly due to a higher acetate production. Taken together, these results suggest the potential prebiotic of hesperidin, nevertheless, further studies are needed to clarify these effects and to elucidate the optimal dosage and food matrix.

3. EXERCISE, FLAVONOIDS AND IMMUNE SYSTEM

Recently, flavonoids have been proposed as ergogenic aids. Moreover, the antioxidant, immunomodulatory and anti-inflammatory properties of flavonoids may be useful in the athletic field to prevent exercise-induced immune disruption. The article below reviewed the clinical studies published between 2005 and 2020 evaluating the role of flavonoids in exercise performance in association with immune function. This systematic review has been published in 2021 in the journal *Nutrients* and includes some of the results obtained in this thesis.

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Review

Does Flavonoid Consumption Improve Exercise Performance? Is It Related to Changes in the Immune System and Inflammatory Biomarkers? A Systematic Review of Clinical Studies since 2005

Patricia Ruiz-Iglesias [1,2], Abril Gorgori-González [1], Malén Massot-Cladera [1,2], Margarida Castell [1,2,3,*] and Francisco J. Pérez-Cano [1,2]

1 Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), 08028 Barcelona, Spain; patriciaruiz@ub.edu (P.R.-I.); abrilgo2008@gmail.com (A.G.-G.); malen.massot@ub.edu (M.M.-C.); franciscoperez@ub.edu (F.J.P.-C.)
2 Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), Universitat de Barcelona, 08921 Santa Coloma de Gramenet, Spain
3 Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain
* Correspondence: margaridacastell@ub.edu; Tel.: +34-93-402-4505

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Abstract: Flavonoids are attracting increasing attention due to their antioxidant, cardioprotective, and immunomodulatory properties. Nevertheless, little is known about their role in exercise performance in association with immune function. This systematic review firstly aimed to shed light on the ergogenic potential of flavonoids. A search strategy was run using SCOPUS database. The returned studies were screened by prespecified eligibility criteria, including intervention lasting at least one week and performance objectively quantified, among others. Fifty-one studies (54 articles) met the inclusion criteria, involving 1288 human subjects, either physically untrained or trained. Secondly, we aimed to associate these studies with the immune system status. Seventeen of the selected studies (18 articles) assessed changes in the immune system. The overall percentage of studies reporting an improved exercise performance following flavonoid supplementation was 37%, the proportion being 25% when considering quercetin, 28% for flavanol-enriched extracts, and 54% for anthocyanins-enriched extracts. From the studies reporting an enhanced performance, only two, using anthocyanin supplements, focused on the immune system and found certain anti-inflammatory effects of these flavonoids. These results suggest that flavonoids, especially anthocyanins, may exert beneficial effects for athletes' performances, although further studies are encouraged to establish the optimal dosage and to clarify their impact on immune status.

Keywords: anthocyanins; cytokines; exhaustion; flavanols; inflammation; quercetin; upper respiratory tract infections

1. Introduction

Among the bioactive compounds provided by diet, flavonoids are one of the most important, given that they are the most abundant polyphenols, regularly ingested in small quantities in many edible plants. Flavonoids are a broad class of secondary plant metabolites with low molecular weight and a flavan nucleus. Chemically, they are benzo-γ-pyrone derivatives consisting of a 15-carbon skeleton arranged in three rings (A, B, and C) (Figure 1). Depending on the chemical structure (hydroxylation pattern, conjugation between the aromatic rings, glycosidic moieties, and methoxy groups); degree of oxidation; and unsaturation of the linking chain, flavonoids are classified into flavanols, flavones, flavonols, flavanones, isoflavones, and anthocyanins (Figure 1) [1].

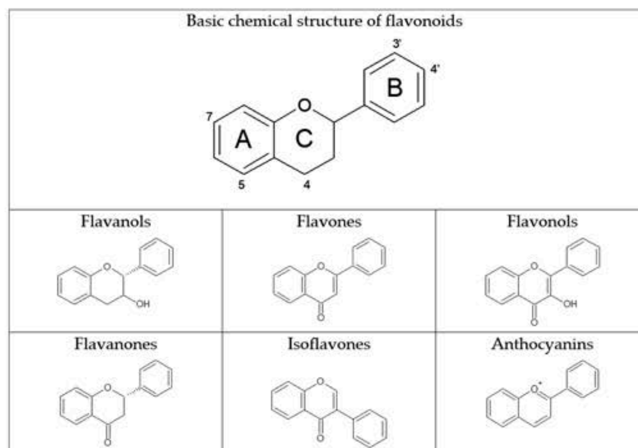

Figure 1. Chemical structures of flavonoids and their classes. Based on reference [1].

Flavonoids comprise more than 4000 compounds that are widely distributed in seeds, leaves, bark, and flowers of plants. Flavanols, as those found in green tea and cocoa, include monomers such as epicatechin, catechin, gallocatechin, epigallocatechin (EGC), and epigallocatechin gallate (EGCG) and, also, polymers called proanthocyanidins or condensed tannins. Flavones are commonly found in fruit skins, parsley, and celery and include glycosides of luteolin, chrysin, and apigenin. Flavonols can be found in onions, apples, berries, leeks, broccoli, blueberries, red wine, and tea and include, among others, quercetin, kaempferol, morin, rutin, myricetin, isorhamnetin, and isoquercetin. Flavanones are exclusive to citrus fruits and can be hesperidin, naringenin, and eriodictyol. Leguminous plants such as soy and soy products contain isoflavones such as genistein and daidzein. Finally, anthocyanins are provided by red wine and berry fruits, such as cherries, strawberries, raspberries, barberries, blueberries, and raisins, and include pelargonidin, cyanidin, and malvidin [1,2].

Flavonoid dietary intakes vary considerably among countries and cultures. It seems to be the major polyphenol class consumed by European adolescents (representing 75-76% of the total polyphenols intake), especially the flavanol and flavanone subclasses [3]. The daily consumption of flavonoids is estimated to be 313.26 mg in Spain [4], 506 mg in France [5], 403.5–525 mg in Poland [6], and 103 mg and 80 mg in Finnish women and men, respectively [7]. Flavonoids possess antioxidant and chelating abilities. Related to these or other properties, flavonoid intakes have been associated with numerous health-promoting physiological benefits for cardiovascular disease, cancer, neurological disorders, aging, obesity, etc. [8–15]. For this reason, a wide range of human intervention studies has been developed, and the results offer promising applications in the prevention of several disorders.

With regard to sports performances, many human [16–19] and animal studies [20,21] have focused on the effect of flavonoids on several outcomes of exercise. Much of this research has studied their protective effects against the oxidative stress associated with physical activity and sports [19,22–24]. It is known that intense physical activity induces changes in the oxidative system of the body, leading to an overproduction of reactive oxygen species (ROS) that may disrupt the physiological balance between ROS generation and the antioxidant defense systems, producing oxidative stress [25]. Flavonoids like cocoa

flavanols [19,26], green tea flavanols [27,28], and blueberry's anthocyanins [29], among others, have demonstrated some promising success in counteracting exercise-induced oxidative stress due to their antioxidant properties.

The immune system is very sensitive to oxidative stress [30], and its function can be modulated by exercise [31]. There is a general consensus that regular bouts of moderate physical activity provide several health benefits, such as enhancing immune functionality [32–34]. Nevertheless, intense exercise can have detrimental effects on the immune system [32,35,36]. In general, exercise alters the phagocytic and inflammatory functions of macrophages, as well as natural killer (NK) cell functions. A moderate exercise enhances the innate immunity by increasing phagocytic and cytotoxic activities [37–39]. Nevertheless, intensive physical exercise has been associated with an inflammatory response [40] and a mobilization of leukocytes [41]. Eventually, there is a decrease in host defenses that leads to an increased susceptibility to infections, especially upper-respiratory tract infections (URTIs) and gastrointestinal infections, in the days following a bout of intense exercise [42].

Many studies have reported the influence of flavonoid consumption in physical activities, but when looking for the effects on performance, the number of studies decreases. Likewise, few reviews have considered physical performance as a criterion or considered only specific sports or the effects of specific supplements [17,19,43,44]. In the current article, we aimed to perform a systematic and broad (2005–2020) review based on the clinical trials (randomized controlled trials) regarding the intake of flavonoids in physical activity, looking at their influence on physical performances. We considered studies focused on healthy people aged between 18 and 50 years with flavonoid consumption, both in the pure form and as an extract, lasting for at least seven days and that objectively measured physical performances with randomized, controlled, simple, or double-blind designs. Likewise, from the studies selected, we focused on those associating changes induced by the flavonoid intake in physical performances with the immune system status.

2. Materials and Methods

2.1. Data Sources and Search Strategy

The search strategy was predetermined following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [45]. The search of articles was run using the SCOPUS (Elsevier) database. The searched terms were related with flavonoids and exercise. The first searched terms were "flavonoids" AND "exercise". Moreover, to obtain articles that may have been omitted in a general search, the concepts "athlete" OR "marathon" OR "training" OR "endurance" OR "sport" OR "players" OR "fitness" OR "cycling" were used with regards to exercise, AND "polyphenol" OR "flavanone" OR "flavone" OR "flavonol" OR "anthocyanin" OR "isoflavone" OR "catechin" OR "hesperidin" OR "glabridin" OR "quercetin" OR "blackcurrant" OR "cherry" OR "green tea extract" were searched regarding flavonoids. The strategy was first applied in July 2020 and updated on December 17, 2020 and included articles since 2005.

2.2. Data Selection

After running the search strategy, the inclusion and exclusion criteria were applied (Figure 2). The exclusion criteria were: (i) preclinical studies; (ii) not written in the English language; (iii) participants with morbidities (diabetes, hypertension, etc.) or overweight; (iv) conference abstracts or reviews; (v) intervention with polyphenols other than flavonoids; (vi) evaluations of only exercise recovery; and (vii) the study not approved by an Ethical committee.

The studies included in the review met the criteria: (i) healthy people aged between 18 and 50 years (mean age in the study ranging between 18 and 50 years); (ii) the study designs as randomized, controlled trial, or either single or double-blind; (iii) an intervention lasting for at least seven days; and (iv) physical exercise performances objectively quantified by

means of either distance, time, work performed, anaerobic potency, anaerobic threshold, or strength.

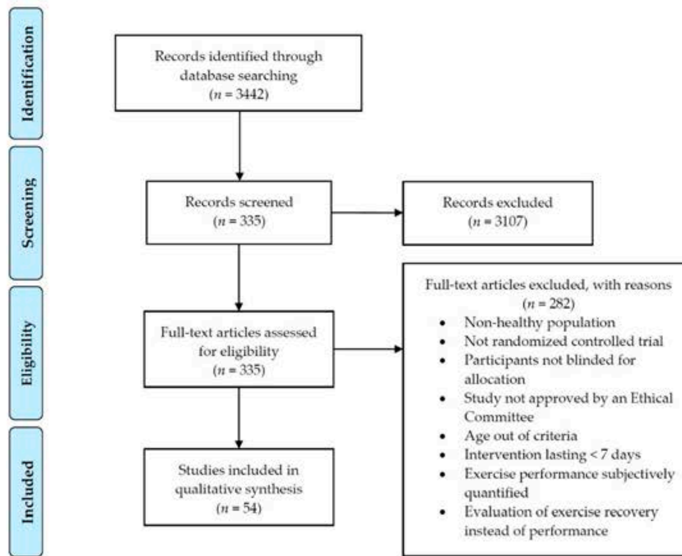

Figure 2. Flow diagram of the article selection process.

2.3. Data Collection

From the selected articles after applying the exclusion and inclusion criteria, the data were collected from the entire paper. Data from (i) the study design, (ii) characteristics of the participants, (iii) flavonoid applied or its composition if there was an extract and dosage used (amount of flavonoid and length of the intervention), (iv) type of exercise, (v) performance outcomes, and (vi) the results and conclusions of the study were collected.

2.4. Assessment of Risk of Bias in Included Studies

The method used for assessing the risk of bias in individual studies was the Cochrane Handbook Guidelines [46]. The domains assessed were selection bias (random sequence generation and allocation concealment), performance bias (blinding of participants and personnel), detection bias (blinding of outcome assessment), attrition bias (incomplete outcome data), reporting bias (selective reporting), and other sources of bias. Each domain was categorized into "low-risk", "high-risk", or "unclear risk" if there was insufficient information to permit the judgment of low or high. Low-risk is interpreted as plausible bias unlikely to seriously alter the results, high-risk is interpreted as plausible bias that seriously weakens the confidence in the results, and unclear risk is interpreted as plausible bias that raises some doubts about the results.

3. Results

3.1. Study Selection

A total of 3442 articles was the result of running the search strategy reported (Figure 2). These articles were screened according to the title and the abstract to apply the exclusion

criteria. As a result, 335 articles could be included. However, after applying the inclusion criteria, 54 articles remained. Figure 3 summarizes the included studies classified according to the flavonoid subclass and the effect on exercise performance.

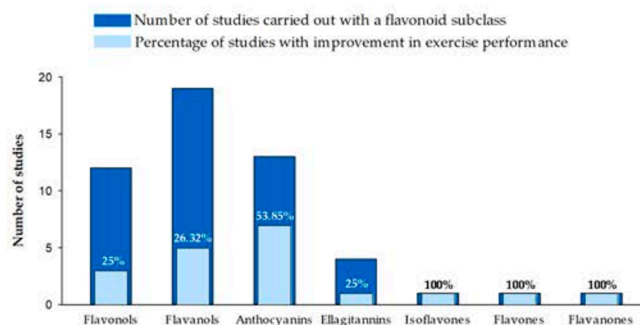

Figure 3. Summary of the included studies classified according to the flavonoid subclass and the percentage of studies reporting improvements in exercise performance.

3.2. Study Characteristics

The studies considered were classified into two categories: those that considered a pure flavonoid (Table 1) and those that included studies carried out with extracts containing flavonoids (Table 2).

3.2.1. Studies with a Single Flavonoid Supplement

From 2005 to 2020, 16 articles, referring to 14 clinical studies, were considered interventions with a single flavonoid administered in the pure form or as a combination with other compounds (Table 1). From these 16 articles [47–62], quercetin was used in 14 papers [47–52,55–62], with two articles by Nieman et al. [52,59] referring to the same clinical trial and two articles by Askari et al. [49,51] also focused on the same participants. Moreover, epicatechin [53] and hesperetin-7-O-rutinoside [54] were used in the other two studies.

- Studies with a Quercetin Supplement

Focusing on the 14 selected articles (12 clinical trials) using quercetin [47–52,55–62], all of them were randomized controlled trials and double-blinded, except for one that was single-blinded [58]. Six studies [47,48,55–58] had a crossover study design, while the six remaining [49,50,59–62] had a parallel design. The included studies involved 382 participants, of whom 335 were males. In eight of these studies [47–50,55–57,62], the participants' mean age was between 19 and 23 years old, in three [58,59,61], it was between 26 and 30 years old, and, in one [60], the mean age was around 45 years old.

Quercetin was mainly administrated in its monomeric form, and only one trial used a glycoside [56]. In 10 studies [47,48,50,55–57,59–62], quercetin was administrated with other compounds, such as vitamin C, tocopherols, green tea, and isoquercetin, among others. The dosage was 1000 mg per day, except in one study that used 500 mg/day [49]. The lengths of the interventions ranged between one and eight weeks. The exercise programs differed among studies: running was chosen in four studies [49,55,56,60]; cycling in four [47,48,59,63]; eccentric contractions in two [50,58]; and, in two trials, running, cycling, and strength exercises were combined [57,62]. The outcomes included distance [55], time [48,56,57,60,63], work performed [47], mean power [59], strength [50,58], or a mix-

ture [49,62]. Some studies verified the absorption of quercetin by measuring its levels in the blood [47,52,55–57,59,60,62], whereas no paper reported harmful effects of this supplement.

From the 14 articles (12 clinical trials) focused on the effects of quercetin on exercise performance, three (25%) reported beneficial effects due to the flavonoid intake, and the training used to establish this effect was cycling [48], running [55], and eccentric contractions [58]. In particular, 12 volunteers (five female and seven male) taking 1000 mg/day of quercetin dissolved in vitamin-enriched Tang for seven days underwent a substantial increase in ride time to fatigue, which was associated with a modest but significant increase in VO_{2max} [48]. Similarly, 26 physically sedentary untrained male adults were supplemented with 1000 mg/day of quercetin for two weeks and were submitted to a 60-min exercise preload and a 12-min running trial on a 15% treadmill grade with self-selected speed [55]. While no significant difference was observed in the first exercise, in the 12-min trial, the distance achieved was significantly greater during the quercetin than the placebo condition. Nevertheless, this improvement was accompanied by a trend to increase the expression of genes related to skeletal muscle mitochondrial biogenesis, which provides a partial explanation for the performance enhancement [55]. Recently, Bazzuchi et al. [58] reported that 12 young men who completed a comprehensive neuromuscular evaluation before, during, and after an eccentric protocol able to induce severe muscle damage showed a higher isometric strength in a maximal voluntary isometric contraction, as well as a lower force and muscle fiber conduction velocity decay during the eccentric exercise, after ingesting 1000 mg/day of quercetin for 14 days compared to the placebo condition. From these results, the authors suggest that quercetin, by an unknown mechanism, can attenuate the severity of muscle weakness caused by eccentric-induced myofibrillar disruption and sarcolemmal action potential propagation impairment [58].

Nevertheless, other studies involving exercise with eccentric contractions were not successful in evaluating the effect of quercetin supplements on exercise performances. O'Fallon et al. [50] analyzed the effects of quercetin supplements in an eccentric exercise and found no differences in the muscle strength and the arm angle after seven day of daily supplementation. Likewise, the evaluation of other markers altered by muscle damage, such as muscle soreness, arm swelling, and the plasma creatine kinase (CK) levels, were not modified by this flavonol.

Other studies focused on the effect of quercetin in running reported nonsignificant effects when a supplement was given for a week [56] or even for a longer period [49,51,60,62]. Abbey et al. [56] reported that the intake of quercetin-3-glucoside for seven days induced a greater percentage of fatigue decrease than the placebo in the repeated-sprint performances of team sports-trained athletes, whereas the blood interleukin (IL) 6, xanthine oxidase activity, and uric acid (related to oxidative stress) was not improved by the flavonoid supplement. The study by Sharp et al. [57] also found no difference induced by quercetin intake in eight-and-a-half days for the oxygen consumption (VO_2 peak and respiratory exchange ratio) of physically active soldiers. Similarly, and for an even longer period, Nieman et al. [60] administered 1000 mg/day of quercetin for three weeks to 18 ultramarathoners (21 for placebo) before competing in the 160-km Western States Endurance Run and observed no significant effects due to the flavonoid supplement in the race time. Moreover, quercetin was not able to attenuate muscle soreness, inflammation, or the increase in cortisol levels, among the other biomarkers experienced by the ultramarathoners [60]. Using a longer time (eight weeks), Askari et al. [49,51] also reported no significant difference in the performances of runners or in the oxygen consumption (VO_{2max}), CK, and aspartate transaminase (markers of muscle damage). Finally, in another study [62], quercetin in the form of four chews (two with breakfast and two with dinner), each one containing 250 mg of quercetin, 100 mg of isoquercetin, 100 mg of omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid (EPA and DHA, respectively)), 30 mg of EGCG, a vitamin mixture, sucrose, and other ingredients in a carnauba wax and soy lecithin base was administered for six weeks to 58 moderately trained men and women. Such a quercetin dosage had no significant effects in four physical performance measures (army physical fitness

test, Baumgartner modified pull-up test, Wingate anaerobic test, and the 36.6-m sprint) or in the VO_{2peak}.

With regard to cycling performances, although Davis et al. found significant improvements with 1000 mg/day of quercetin for seven days [48], Cureton et al., using a double-blind, pretest–post-test control group design with 30 recreationally active, but not endurance-trained, men after 1000 mg/day of quercetin supplement for 7–16 day, showed no improvements in the total work done during the 10-min maximal-effort cycling trial, the phosphocreatine recovery time constant, VO_{2peak}, substrate utilization, or perception of effort during the submaximal exercise [47]. Using a longer supplementation period (three weeks), Nieman et al. [52,59], found no effects for quercetin applied to 40 cyclists after a three-day intensified exercise period (nine h of exercise) in either the changes in plasma cortisol, epinephrine, and norepinephrine levels or in immune function biomarkers, as stated in Section 3.4. On the other hand, Nieman et al. [61] also studied the effect of a two-week 1000 mg/day of quercetin, 1000 mg/day of vitamin C, 40 mg/day of niacinamide, and 800 µg/day of folic acid supplementation (1000 mg/day) and its combination with 120 mg of EGCG, 400 mg of isoquercetin, and 400 mg of polyunsaturated fatty acids (PUFAs, EPA, and DHA) on three cycling time trial performances (5-, 10-, and 20-km time trials carried out on consecutive days); mitochondrial biogenesis; immunity; or inflammation. No changes were found for the time trial finish time and cycling power output or in the mRNA expression for the gene's peroxisome proliferator-activated receptor γ coactivator α, citrate synthase, and cytochrome c, which are related to muscle mitochondrial biogenesis. However, a two-week supplementation of quercetin and EGCG, among other nutrients, resulted in a greater granulocyte oxidative burst at the baseline and a decrease in the inflammatory and immune biomarkers, as commented on below (Section 3.4), immediately after the third exercise bout [61].

- Studies with other Pure Flavonoids

In recent years, two interesting papers that examined the effects of other pure flavonoids on the cycling performance appeared (Table 1). Schwarz el al. [53], in a double-blind, randomized, placebo-controlled parallel study, applied 200 mg/day of (–)-epicatechin to 20 recreationally active men and women in anaerobic and aerobic cycling conditions. The flavanol administration inhibited the development of a peak relative aerobic power and conferred no additional benefit for the peak anaerobic power or anaerobic capacity when compared to the placebo.

On the other hand, Overdevest et al. [54], in a randomized, parallel-group, double-blind design, administered 500 mg of citrus flavonoid extract with hesperetin-7-O-rutinoside 2S enantiomer (with a total rutinoside content of at least 90%) to 39 cyclists. They found that this flavanone increased both the absolute and relative power output in a 10-min time trial on a cycle and decreased the oxygen consumption/power ratio.

Table 1. Summary of the included studies assessing the effects of a single flavonoid intervention on exercise performances.

Reference	Flavonoid	Control Group	Study Design	Number of Participants (Female + Male)	Mean Age of the Participants (Years)	Dosage	Exercise	Performance Variable	Effect
Quercetin Supplements									
[52,59]	Quercetin + Tang powder	Tang powder	Db RPCT	0 + 40	26.1 ± 1.8 (SUP) 29.1 ± 2.4 (PL)	1000 mg/d for 3 wks	Three 3 h cycling bouts	Mean power	NS
[61]	Quercetin + isoquercetin + EGCG	Placebo +/− Quercetin	Db RPCT	7 + 32	26.3 ± 1.7 (PL) 26.8 ± 2.6 (Q) 28.1 ± 2.8 (Q + EGCG)	1000 mg quercetin + 120 mg EGCG + 400 mg/d isoquercetin for 14 d	Cycling	5, 10 and 20 km time trials	NS
[47]	Quercetin + sport hydration beverage	Sports hydration beverage	Db RPCCT	0 + 30	23.1 ± 2.4 (SUP) 22.1 ± 1.8 (PL)	1000 mg/d for 7–16 d	Cycling	Work performed in a 10 min maximal effort cycling	NS
[48]	Quercetin + Tang	Tang	Db RPCCT	5 + 7	22.9 ± 2.4	1000 mg/d for 1 wk	Cycling	Time to fatigue	Improvement
[60]	Quercetin + vit C + niacin	Placebo chews	Db RPCT	7 + 32	44.2 ± 2.0 (SUP) 46.0 ± 2.3 (PL)	1000 mg/d quercetin + 1000 mg/d vit C + 80 mg/d niacin for 3 wk	160-km Western States Endurance Run	Race time	NS
[55]	Quercetin + PowerAde Coca Cola	PowerAde Coca Cola	Db RPCCT	0 + 26	20.2 ± 0.4	1000 mg/d for 14 d	12 min running trial	Distance	Improvement
[62]	Quercetin + Isoquercetin + EGCG + Vit mix + EPA and DHA	Placebo chews	Db RPCT	14 + 44	22.0 ± 5.1 (SUP) 20.3 ± 1.6 (PL)	1000 mg/d for 6 wks	APFT, BMPU, WAnT, and 36.6 m running sprint	Time trial, repetitions, mean power and time trial	**NS**

Table 1. *Cont.*

Reference	Flavonoid	Control Group	Study Design	Number of Participants (Female + Male)	Mean Age of the Participants (Years)	Dosage	Exercise	Performance Variable	Effect
[56]	Quercetin-3-glucoside + 6% carbohydrate sports drink	6% carbohydrate sports drink	Db RPCCT	0 + 15	23.3 ± 2.6	1000 mg/d for 1 wk	Running repeated sprints	Mean sprint time	NS
[57]	Quercetin + food bars	Energy bars	Db, RPCCT	0 + 16	22.0 ± 3.0	1000 mg/d for 8.5 d	Marching in a treadmill and cycling trial	Time trial	NS
[49,51]	Quercetin	Placebo +/- vit C	Db, RPCT	0 + 65	21.0 ± 1.6	500 mg/d for 8 wks	Running in a treadmill	Time to exhaustion or distance covered	NS
[50]	Quercetin + vit C + tocopherols	Energy bars containing vit C and tocopherols	Db, RPCT	14 + 16	19.6 ± 1.3 (female PL) 20.6 ± 1.1 (female SUP) 19.5 ± 1.1 (male PL) 20.9 ± 1.8 (male SUP)	1000 mg/d quercetin + 20 mg/d vit C + 14 mg/d tocopherols for 1 wk	Eccentric contractions of the elbow flexors	Muscle strength, arm angle	NS
[58]	Quercetin	Placebo capsules	RPCCT	0 + 12	26.1 ± 3.1	1000 mg/d for 14 d	Eccentric contractions	Arm angle, arm circumference	Improvement
Other Flavonoid Supplements									
[53]	(-)-epicatechin	Cellulose capsules	Db, RPCT	20	20.5 ± 1.5 (SUP) 21.0 ± 1.9 (PL)	200 mg/d for 4 wks	Cycling	Peak anaerobic power	Worsening
[54]	Hesperetin-7-O-rutinoside	Microcrystalline cellulose capsules	Db, RPCT	0 + 39	23.0 ± 0.3	500 mg/d for 4 wks	Cycling	Absolute power output	Improvement

Db = double-blind, RPCT = randomized placebo-controlled trial, RPCCT = randomized placebo-controlled crossover trial, PL= placebo, SUP = flavonoid-supplemented, d = day, wk = week, NS = nonsignificant effect, EGCG = epigallocatechin gallate, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, APFT = Army Physical Fitness Test, BMPU = Baumgartner Modified Pull-Up Test, Vit = vitamin, and WAnT = Wingate Anaerobic Test.

3.2.2. Studies with Flavonoid-Enriched Extracts

As shown in Table 2, since 2005, 38 articles have reported the effects of flavonoid-enriched extracts on sports performances [26,28,64–99], mainly using cycling and running performances but, also, strength testing, vertical jump, taekwondo, and the climbing test. These 38 articles referred to 37 clinical trials, because the articles by Nieman et al. [97] and Ahmed et al. [98] used the same clinical trial. Eighteen clinical trials applied extracts containing flavanols, 13 applied anthocyanins, 4 used ellagitannins, and flavones and isoflavones were each used in one. The length of the intervention ranged between seven days and eight weeks. From these studies, 41% (15/37) reported an improved performance after the flavonoid intake. This positive effect was obtained for 28% (5/18) studies using flavanols and about 54% (7/13) testing anthocyanins (Figure 2). The remaining improvements were observed with extracts containing ellagitannins (one out of four), isoflavones (one out of one), and flavones (one out of one).

- Studies with Flavanols

A total of 19 articles [26,28,64–72,74,85,94–99] referred to 18 clinical trials that applied extracts rich in flavanols as the intervention (Table 2, flavanols section). Two of these 19 articles focused on the same clinical trial [97,98]. Twelve clinical trials had a parallel design [64–67,70–72,94,96–99], and the six remaining had a crossover design [26,28,69,74,85,95]. Although most of the studies were double-blind, one trial was single-blind [26] and two were triple-blind [64,68]. There was a total of 495 participants involved in these studies, of whom 35 were women. Most of the studies were performed on a young population, with a mean age of between 19 and 25 years; in six studies, the participants were between 25 and 35 years [68,69,71,85,95,97,98], and in two, they were over 36 years [67,74].

The flavanol sources differed among the studies. Green tea extract was the most common [28,64,85,94–96,99], followed by chocolate [26,68–71], lychee [65,66], apple [74], carob [72], and three combined green tea extracts with anthocyanins [67,97,98]. The dosage and the length of the intervention also varied between studies. In nine studies, the dosage was less than 250 mg [26,65–69,72,85,96], three between 251 and 500 mg [64,70,71], two between 501 and 750 mg [94,95], one 751 and 999 mg [28], and four more than 1000 mg [74,97–99]. The lengths of the interventions ranged between one and ten weeks. The exercise protocols included running [65–67,71,96–98], cycling [26,28,68,69,74,85,94,95], strength [99], the calf-raising exercise [64], vertical jump [70], and taekwondo [72].

Flavanols were successful in enhancing performances after cycling (three out of eight reviewed studies reported ergogenic effects in cycling) [74,94,95], running (one out of six) [66], or taekwondo training (one out of one) [72]. The length of the successful interventions varied between seven days and six weeks. Ataka et al. [74] demonstrated the positive effect of the seven-day intake of Applephenon®, which contained procyanidins as the active components. In particular, 18 volunteers were asked to perform non-workload trials with a maximum velocity for 10 s at 30 min (30-min trial) after the start and 30 min before the end (210-min trial). The change in maximum velocities between the 30- and 210-min trials was higher in the Applephenon® group than in the placebo group [74]. Similarly, Roberts et al. [94] showed the beneficial effects of a four-week administration of a decaffeinated green tea extract containing 70% EGCG (i.e., 400 mg/day) in 14 volunteers performing one hour at 50% VO_{2peak} cycling. The use of the decaffeinated green tea extract resulted in an increase in distances covered at two and four weeks of the intervention. This effect was accompanied by a higher total fat oxidation rate and a decrease in the body fat index, although the total fatty acids concentration was unaffected [94]. Similarly, Ota et al. [95] applied a green tea extract that provided 570 mg of catechins in a longer (eight weeks) study. Fourteen untrained volunteers underwent cycle exercise training twice a week during the eight-week period, and at the end, their isokinetic muscle strength, among other variables, were measured. The supplement increased their leg extension strengths without changing their muscle mass, and, in addition, there was an increase in their aerobic endurance capacities (ventilation threshold) [95].

Likewise, Kang et al. [66] demonstrated the influence of an oligomerized lychee extract (Oligonol®), 200 mg/day for 30 days, in a running capacity. The extract contained 15.7% flavanol monomers ((+)-catechin, and (−)-epicatechin), along with 13.3% flavanol dimers (procyanidin B2, etc.), and was able to elevate the submaximal running time and increase the anaerobic threshold when compared to the baseline values. On the other hand, Gaamouri et al. [72] applied a carob extract, which is rich in carbohydrate, dietary fiber, and polyphenols, in taekwondo athletes. In particular, carob extract is rich in flavanols such as (+)-catechin, (−)-epicatechin, (−)-epicatechingallate, EGC, EGCG, and condensed tannins and also contains considerable amounts of other polyphenols (i.e., gallic acid) [100]. After eight weeks of supplementation and training, the athletes that took the carob extract improved their distance and the maximal aerobic velocity compared to those taking the placebo after performing a final Yo-Yo intermittent recovery test level-1. The study also demonstrated that the supplement increased the weight loss of athletes [72].

Nevertheless, not all interventions with extracts containing flavanols revealed a higher performance, either applied in cycling [26,28,68,69,85,101], running [65,67,71,96–98] or other protocols [64,70,99]. Eichenberger et al. [85] analyzed the effects of a green tea extract (159 mg/day catechins) for three weeks in nine endurance-trained men who cycled for two h and then performed a 30-min time trial. Although no improvements in their performances, or in fat oxidation and energy expenditure, were obtained, the supplementation reduced some inflammation biomarkers, as stated below (Section 3.4.). A green tea extract in cycling performances was also applied in the study by Jówko et al. [28]. In a study including four weeks of administration of green tea extract providing 900 mg/day of catechins, the authors demonstrated that 16 sprinters who performed two repeated-cycle sprint tests did not improve their sprint performances through taking green tea extract. However, the supplement prevented the increase in blood biomarkers of oxidative stress [28].

Supplements of flavanol-enriched foods were also applied by means of dark chocolate. Allgrove et al. [26] assessed the effects of two weeks of consumption of dark chocolate in 20 active men cycling at 60% VO_{2max} for 1.5 h, with the intensity increased to 90% VO_{2max} for a 30-s period every 10 min, followed by a ride to exhaustion at 90% VO_{2max}. Although dark chocolate provided 197.7 mg/day of flavanols (108.6 mg of monomeric forms and 88.8 mg of procyanidins), the time to exhaustion in the final cycling test did not differ between the supplement conditions. Nevertheless, dark chocolate consumption decreased the plasma levels of the oxidative stress biomarkers [26]. Similarly, Decroix et al. [68] studied the effects of cocoa flavanols on 14 trained cyclists in a randomized, double-blind, crossover study. The daily intake of cocoa flavanols (1765 mg/day of cocoa extract with 121 mg/day of monomeric forms) for seven days reduced the oxidative stress but did not improve the exercise performance during exhaustive exercise in hypoxia. More recently, Shaw et al. [69] also studied the effects of chocolate flavanol intake for two weeks in trained cyclists at altitude. In agreement with Decroix et al. [68], dark chocolate had no effect on the cycling performances.

With regards to flavanols in running, various studies showed no improvements in running performances. Nishizawa et al. [65] applied a lychee fruit extract (100 mg/day) containing monomers (16.3%), dimers (13.8%), trimmers (3.8%), and larger proanthocyanidins (58.6%) throughout the two-month training period of long-distance runners. The lychee fruit extract did not improve the time for a five-km track race [65]. Additionally, using long-distance runners, Scherr et al. [67] studied the effects of drinking nonalcoholic beer for three weeks, consisting predominantly of catechin, epicatechin, procyanidin B-3, and flavonols, in athletes who participated in the Munich Marathon 2009. No difference was observed in the time trial between those supplemented with flavonoids and those receiving the placebo. Nevertheless, the supplement decreased some immune outputs, as stated below (Section 3.4). The Nieman et al. group [97,98] could not demonstrate the efficacy of a mix of water-soluble polyphenols from blueberry and green tea extracts captured in a polyphenol soy protein complex (40 g/day with about 1 g/day of flavanols for two weeks) in improving the performances of long-distance runners. In addition, this

supplement did not counteract the increase in inflammation (see Section 3.4) and oxidative stress biomarkers, although a distinct gut-derived phenolic signature was found [97]. On the other hand, a green tea extract combined with endurance training was tested in untrained men [96]. The supplement provided 207 mg/day of catechins and was given for four weeks, in which endurance training was performed. The flavanol-enriched extract did not improve the endurance training capacity but protected against acute exercise-induced muscle damage and oxidative stress in sedentary men [96]. More recently, García-Merino et al. [71] reported the effect of 5 g/day of cocoa (425 mg of flavanols, mainly procyanidin B2, with small amounts of flavonols, flavanones, and flavones) for 10 weeks in endurance cross-country athletes. The long intervention decreased the body and visceral fat levels of the athletes, but it did not improve their exercise performances.

Beyer et al. [99] applied a proprietary blend of aqueous tea extracts (2 g/day for four and six weeks) from *Camellia sinensis* (green tea) containing a minimum of 40% polyphenols in untrained men and assessed their lower-body strength. *Camellia sinensis* contains EGCG, ECG, and other monomer flavanols, together with caffeine and, also, minerals such as potassium [102]. Although the four-week green tea extract supplementation increased the antioxidant capacity, six weeks of progressive resistance training showed no difference in the strengths of the supplemented, placebo, and control groups [99]. Similarly, da Silva et al. [64] evaluated the potential of green tea extract (500 mg for 15 days) on the calf-raising exercise and did not find beneficial effects from the supplement either in the number of repetitions, muscle soreness, oxidative damage, or antioxidant status. On the other hand, de Carvalho et al. [70] assessed the effect of chocolate milk with additional cocoa flavanols (308 mg) in rugby players performing vertical jumps and a Yo-Yo test to establish athlete performance. After seven days of supplementation, there were no benefits in athletic performance or in oxidative stress.

- Studies with Anthocyanins

Thirteen studies with anthocyanins were selected for the final discussion (Table 2, anthocyanins section). All of them were randomized controlled trials and double-blinded. Seven studies [73,75,76,78–80,83] had a crossover design, and the other six [77,82–84,86,87] had a parallel design.

Around 80% of the participants were male; specifically, there were 217 males out of 263 participants. In six studies [76,78,79,81,83,84], the mean age was between 19 and 25 years old. There were three studies [77,80,82] with people aged between 26 and 30 years, one [75] between 31 and 35, and three [73,86,87] with subjects aged between 36 and 40.

In eight trials [73,75–81], blackcurrants was the food source of anthocyanins, and, except for one [75], it was specifically New Zealand blackcurrants (CurraNZ™). Three studies [82–84] were performed with Montmorency tart cherries, one [86] with purple grapes, and one [87] with blueberries. The dosage varied among studies between 105 mg/day [73,76] and 10 mL/kg/day, containing 52.6 mg/L of anthocyanins [86]. The most common dosage was between 200 and 300 mg/day [75,77–81,83], with only two studies using a dosage higher than 300 mg/day [87,103]. The length of the intervention in nine studies [73,76–83] was one week, with the four remaining studies being 10 days and two, three, and four weeks, respectively [75,84,86,87]. Running was the most common exercise program among the studies [75–77,84,86], followed by cycling [64,73,82,87] and strength through isometric exercise [78,79], intermittent forearm exercise protocol [79], and climbing [81]. Performance was measured through a time trial [73,75,77,83,84,87], maximal contractions [78,79], distance covered [76], work performed [82], and time to exhaustion [80,81,86].

Anthocyanins were able to improve the running [76,84,86], cycling [73,83], and climbing [81] performances after 7–28 days supplementation. Three studies using New Zealand blackcurrant extract (CurraNZ®) found a better performance after a seven days of supplementation [73,76,81]. One 300-mg capsule of CurraNZ® contains 105 mg of anthocyanins—specifically, 35–50% delphinidin-3-rutinoside, 5–20% delphinidin-3-glucoside, 30–45% cyanidin-3-rutinoside, and 3–10% cyanidin-3-glucoside. Cook et al. [73] reported a 2.4%

improvement in a 16.1-km cycling time trial following 30 min of steady-state cycling (3 × 10 min at 45%, 55%, and 65% VO_{2max}) after seven days of intake of CurraNZ® (one capsule/day). The New Zealand blackcurrant supplementation also increased the fat oxidation during cycling at 65% VO_{2max} and plasma lactate immediately after completing the time trial [73]. Perkins et al. [76] also demonstrated the beneficial effects on performance of the seven days of intake of CurraNZ® (one capsule/day). Specifically, 13 active men performed a high-intensity intermittent running protocol on a treadmill, which consisted of combining 6 × 19 s of sprints and 15 s of low-intensity running. The total distance running and the distance covered during the sprints increased by 10.6% and 10.8%, respectively, after the seven-day New Zealand blackcurrant supplementation. Moreover, the post-exhaustion blood lactate levels tended to be higher after the blackcurrant intake [76]. Likewise, Potter et al. [81] showed the positive effects of seven days of CurraNZ® supplementation using a higher dose (2 × 300 mg CurraNZ® capsules/day, providing a total of 210 mg/day of anthocyanins) on sports climbing ability. Participants performed three climbing bouts separated by a 20-min recovery period; in each of which, they had to climb without stopping until volitional exhaustion. After supplementation, the total climbing time was increased by 23%, and the decline in climbing performance observed in the placebo condition across the repeated climbing bouts was avoided, whereas no effect was found regarding the hang time and pull-ups. No changes were observed in heart rate, blood lactate, forearm girth, or handgrip strength due to seven days of blackcurrant intake [81].

On the other hand, Braakhuis et al. [75] studied the effects of an antioxidant drink that combined blackcurrant extract and a fruit drink concentrate, providing 300 mg of anthocyanins and 15 mg of vitamin C, on training and performance in trained female runners. Participants drank 0.5 L of the antioxidant juice daily for three weeks; during which, they trained two to three times a week according to their fitness level under the supervision of one of the researchers. At the end of the supplementation period, the participants performed a 5-km time trial on a treadmill followed by an incremental running test, in which the speed and inclination were progressively increased until exhaustion. The results are quite controversial, since, whereas the fastest runners (+2 standard deviation of the mean speed on the incremental running test) showed an improved running performance in both the 5-km time trial and the incremental running test after supplementation, the average runners tended to be slower after the three weeks of intervention [75].

Despite these successful results regarding blackcurrant extract, other authors did not find changes in sports performance with a similar flavonoid source. This was the case in studies that focused on the half-marathon finish time [77], isometric maximal voluntary contractions of the quadriceps [78], and time to exhaustion during repeated intermittent forearm muscle contractions [79,80], which did not improve due to seven days of intake of 600 mg/day of New Zealand blackcurrant extract (two capsules/day of CurraNZ®).

On the other hand, three articles studied the effects of anthocyanins from Montmorency tart cherries on running [84] and cycling [64,82] performances. Levers et al. [84] observed a 13% reduction in the half-marathon finish time due to a seven-day supplementation with 480 mg/day of Montmorency tart cherry powder (CherryPURE®, Traverse City, MI, USA), providing 991 mg of phenolic compounds and 66 mg of anthocyanins. Moreover, the tart cherry supplementation also avoided the cortisol production increase observed in the placebo condition 60 min after exercise and attenuated some of the changes induced by exercise on the muscle catabolic markers and inflammatory markers, as stated in Section 3.4 [84]. Similarly, Morgan et al. [83] observed a 4.6% decrease in the time needed to complete a 15-km cycling time trial after a 10-min steady-state cycling at 65% VO_{2max} due to a seven-day Montmorency tart cherry supplementation (CherryActive® capsules, Hanworth, UK), providing 462.8 mg/day of polyphenols and 256.8 mg/day of anthocyanins. This improvement in cycling performance was accompanied by an increase in the baseline tissue oxygenation index and a higher blood lactate concentration at the end of the steady-state exercise [83]. Bell et al. [82] also studied the effect of a seven-day Montmorency

tart cherry supplementation (60 mL/day of CherryActive® concentrate juice, containing 9.117 mg/mL of anthocyanins) on the cycling time trial performance; however, they found no difference in the total work performed during the time trial due to the intervention. Although the supplementation did not alter the performances, Montmorency tart cherry supplementation attenuated the exercise-induced increase in lipid hydroperoxides and the inflammatory response [82].

Additionally, other anthocyanin-rich extracts have been studied in recent years. In this regard, Toscano et al. [86] showed the ergogenic effect of a 28-day integral purple grape juice intake (10 mL/kg/day containing 1.82 g/L of total phenolic compounds and 52.58 mg/L of anthocyanins) in recreational men and women runners. A 15.3% increase in the time to exhaustion was accompanied by an increased total antioxidant capacity and a higher serum content of vitamin A and uric acid, as well as a decrease in the inflammatory biomarker α-1-acid glycoprotein serum concentration [86]. On the other hand, Nieman et al. [87] analyzed the effects of a two-week freeze-dried blueberry supplementation (one cup/day blueberries equivalent, providing 345 mg of anthocyanins) and its acute combination with banana as a carbohydrate source (banana) during exercise on a 75-km cycling time trial performance and stressful exercise-induced oxylipins production. Although no significant differences in the cycling power or finish time during the time trial were found, the two weeks of blueberry intake increased the blood levels of some gut-derived phenolic metabolites [87].

- Studies with Ellagitannins

Four studies included in this systematic review used extracts enriched in ellagitannins (Table 2, ellagitannins section). All studies were double-blind, and three [88–90] had a crossover design, whereas one [91] was parallel. There was a total of 63 participants, and only two were women. The average ages of the participants ranged between 20 [91] and 37 [89] years old.

All studies on ellagitannins used pomegranate as the food source. The dosage was 171.9 mg/day [88], 220 and 225 mg/day [90,91], or 11.46 mg/kg/day [89]. In two of the studies, the length of the intervention was one week [88,89], whereas another lasted two weeks [90], and the fourth lasted two months [91]. Performances were measured in cycling trials through a time trial [88], average power output [89], time to exhaustion [90], or time to complete 2000 m on a rowing ergonometer [91].

Improvements in cycling performances were reported by Torregrosa-García et al. [90] in 26 amateur trained cyclists after 15 days of supplementation with POMANOX® P30, providing 225 mg/day of punicalagins α and β. During the intervention period, participants had a training routine of two–four sessions per week, each session lasting at least one hour. At the end of the supplementation period, participants were submitted to an incremental exercise test to exhaustion on a cycle ergometer, in which the total time to exhaustion and time to reach ventilatory threshold 2 (previously established before starting supplementation) were greater after the 15 days of pomegranate intake. Moreover, the authors also evaluated the effects of these ellagitannins on force recovery through a repeated isokinetic unilateral leg test performed 2, 24, 48, and 72 h after inducing muscle damage through an eccentric drill protocol; however, no significant changes were found [90]. On the other hand, Trinity et al. [88] and Crum et al. [89] found no impacts of the seven-day and eight-day pomegranate ellagitannin intakes, respectively, on the cycling time trials. Finally, the Polish rowing team was used to establish the effect of a two-month pomegranate juice intake (220 mg/100 g of polyphenols) on the rowing performance, antioxidant potential, and markers of iron metabolism [91]. No differences were found in the power output and total row time over a 2000-m distance due to supplementation or on iron metabolism markers. However, they found a higher total antioxidant capacity in pomegranate-supplemented rowers one day after the 2000-m rowing test [91].

- Studies with Other Flavonoids

Two clinical trials applied other flavonoids (Table 2). One of them studied the impact of isoflavone supplementation [92]. For that purpose, 14 men daily consumed four 500-mg

capsules of a proprietary blend, each one containing 150 mg of soybean peptides, 50 mg of taurine, 45 mg of *Purearia Radix* isoflavone, and 30 mg of ginseng saponin complex (STPG capsule), for 15 days. At the end of the supplementation period, the participants carried out an exhaustive cycling test at an intensity of 75% VO_{2max}, where the time to exhaustion was greater in the supplemented group. This improvement in the performance after the 15-day isoflavone intervention was accompanied with higher serum concentrations of nonesterified fatty acids from 15 min of exercise onward, attenuating the decrease observed in the placebo group over the exhaustive test. Moreover, although the plasma lactate increased with exercise in both groups, lower plasma lactate levels were found after 20 and 25 min of exercise in the supplemented one. The ammonia and glycerol plasma levels also increased throughout the exhaustive test in both conditions [92].

Finally, Gelabert-Rebato et al. [93] studied the ergogenic effects of peanut husk extract (PHE) containing 95% of the flavone luteolin in combination with mango leaf extract (MLE) containing 71% of the xanthone mangiferin at low (50 mg/day of PHE and 140 mg/day of MLE) and high (100 mg/day of PHE and 420 mg/day of MLE) doses. The participants performed two exercise protocols after 48 h and 15 days of supplementation in order to assess both the acute and prolonged changes. The exercise protocols involved both low- and high-intensity stages and repeated sprinting bouts in combination with ischemia–reperfusion episodes. The 15-day intake of both tested doses of the luteolin- and mangiferin-rich extracts combination enhanced the sprint performance after ischemia–reperfusion by 22% in terms of the peak power output compared with the first exercise trial performed 48 h after starting the nutritional intervention. No changes in performances were found in the placebo group in either exercise protocols. Moreover, the supplementation with luteolin combined with mangiferin also improved the muscle O_2 extraction and brain oxygenation [93].

Table 2. Summary of the included studies assessing the effects of flavonoid-enriched extracts on exercise performances.

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
Flavanols									
[74]	Apple extract (Applephenon®)	Crystalline cellulose capsules	Db RPCCT	9 + 9	39.1 ± 9.1	720 mg/d procyanidins for 7 d	Cycling	Change of maximum velocity	Improvement
[85]	Green tea extract	Carbohydrate-containing drink	Db RPCCT	0 + 9	32.2 ± 2.1	159 mg/d catechins for 3 wks	Cycling	Time for 30 km trial	NS
[28]	Green tea extract	Microcrystal-line cellulose capsules	Db RPCCT	0 + 16	21.6 ± 1.5	800 mg/d catechins for 4 wks	Cycling	Peak power, mean power, total work output	NS
[94]	Decaffeinated green tea extract	Corn flour capsules	Db RPCT	0 + 14	21.4 ± 0.3	400 mg/d EGCG for 4 wks	Cycling	Distance	Improvement
[95]	Green tea extract	Sports drink	Db RPCCT	0 + 14	33.9 ± 7.4	570 mg/d catechins for 8 wks	Cycling	Leg extension strength	Improvement
[96]	Green tea extract	Starch capsules	Db RPCT	0 + 40	21.0 ± 1.0	207 mg/d catechins for 4 wk	Running	Time to exhaustion	NS
[97,98]	Blueberry-green tea-polyphenol soy protein complex	Soy protein complex with non-polyphenolic food coloring	Db RPCT	13 + 18	33.7 ± 6.8 (SUP) 35.2 ± 8.7 (PL)	1001 mg/d flavanols for 17 d	Running in a treadmill for 2.5 h	Distance covered	NS

Table 2. *Cont.*

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
[99]	Green tea extract	Microcrystalline cellulose capsules	Db RPCT	0 + 40	23.3 ± 4.1 (CT) 21.9 ± 2.5 (SUP) 21.5 ± 2.3 (PL)	800 mg/d polyphenols for 4 wks	Maximal strength testing, lower body resistance training	Strength	NS
[64]	Green tea extract	Celulomax® capsules	Tb RPCT	0 + 20	25 ± 5	18.5 mg/d catechins for 15 d	Calf-rising exercise	Number of repetitions	NS
[65]	Flavanol-rich lychee fruit extract (Oligonol ®)	Malt extract	Db RPCT	0 + 20	20.6 ± 1.3 (SUP) 20.6 ± 1.2 (PL)	100 mg/d flavanols for 2 months	Running training, combining low, medium, and high intensities	Time for 5-km race	NS
[66]	Oligomerized lychee fruit extract (Oligonol®)	Dextrin capsules	Db RPCT	0 + 38	24.6 ± 6.6 (SUP) 22.9 ± 3.6 (PL)	200 mg/d flavanols for 30 d	Running	Submaximal running time	Improvement
[67]	Nonalcoholic beer	Control beverage containing the same ingredients except for polyphenols	Db RPCT	0 + 121	44 (SUP) 42 (PL)	1.0–1.5 L/d with 47 mg/L catechin and 33 mg/L procyanidins for 3 wks	Munich marathon race	Time for the race	NS

Table 2. *Cont.*

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
[26]	Dark chocolate	Isocaloric control chocolate without polyphenols	Sb RPCCT	0 + 20	22.0 ± 4.0	197.4 mg of flavanols for 2 wks	Incremental cycling	Time to exhaustion	NS
[68]	Cocoa flavanols	Maltodextrin capsules containing the same amount of theobromine and caffeine than cocoa flavanols capsules	Db RPCT	0 + 14	30.7 ± 3.1	100 mg epicatechin and 23 mg catechin for 7 d	Cycling trial in normobaric hypoxia	Completed work in 20 min cycling trial	NS
[69]	Dark chocolate	Isocaloric nonchocolate placebo	Db RPCCT	2 + 10	35.0 ± 12.0	60 g/d dark chocolate for 14 d and 120 g just before trial	10 km cycling trial at altitude	Time trial	NS
[70]	Cocoa flavanols	Chocolate milk	Db RPCT	0 + 13	20.69 ± 1.49	308 mg/d flavanols for 7 d	Vertical-jump and yo-yo tests	Vertical jump performance, accumulated distance covered	NS
[71]	Cocoa flavanols	Maltodextrin	Db RPCT	0 + 32	33 ± 7 (SUP) 36 ± 8 (PL)	425 mg/d flavanols for 10 wks	Treadmill running	Time to run 1 km	NS

Table 2. *Cont.*

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
[72]	Carob extract	Carob-flavored commercial drink containing citric acid, sweeteners, and stabilizers	Db RPCT	11 + 12	21.91 ± 1.22	14.4 mg/d flavonoids for 6 wks	Taekwondo training + yo-yo tests	Distance covered, maximal aerobic velocity	Improvement
Anthocyanins									
[73]	New Zealand blackcurrant (CurraNZ™)	Microcrystal-line cellulose capsules	Db RPCCT	0 + 14	38.0 ± 13.0	105 mg/d anthocyanins for 7 d	Cycling trial	Time trial	Improvement
[75]	Blackcurrant juice	Orange flavored sports drink	Db RPCCT	23 + 0	31.0 ± 8.0	300 mg/d anthocyanins for 3 wks	Running test	Time trial	Worse for average runners, improvement for fast runners
[76]	New Zealand blackcurrant (CurraNZ™)	Microcrystal-line cellulose capsules	Db RPCCT	0 + 13	25.0 ± 4.0	105 mg/d anthocyanins for 7 d	Treadmill running	Running distance	Improvement
[77]	New Zealand blackcurrant (CurraNZ™)	Microcrystalline cellulose capsules	Db RPCT	8 + 12	30.0 ± 6.0	210 mg/d anthocyanins for 7 d	Chichester half-marathon	Finish time	NS
[78]	New Zealand blackcurrant (CurraNZ™)	Microcrystal-line cellulose capsules	Db RPCCT	0 + 13	25 ± 4	210 mg/d anthocyanins for 7 d	Submaximal isometric exercise	Isometric maximal voluntary contractions	NS

Table 2. *Cont.*

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
[79]	New Zealand blackcurrant (CurraNZ™)	Microcrystal-line cellulose capsules	Db RPCCT	0 + 12	25.0 ± 4.0	210 mg/d anthocyanins for 7 d	Submaximal forearm muscle contractions	Maximal volitional contraction	NS
[80]	New Zealand blackcurrant (CurraNZ™)	Microcrystal-line cellulose capsules	Db RPCCT	0 + 12	26.0 ± 5.0	210 mg/d anthocyanins for 7 d	Submaximal forearm muscle contractions	Time to exhaustion	NS
[81]	New Zealand blackcurrant (CurraNZ™)	Microcrystal-line cellulose capsules	Db RPCCT	0 + 18	24.0 ± 6.0	210 mg/d anthocyanins for 7 d	Climbing ability test	Time to exhaustion	Improvement
[82]	Montmorency tart cherry concentrate (Cherry Active® concentrate juice)	Commercially cordial with less than 5% fruit, mixed with water and maltodextrin	Db RPCT	0 + 16	30.0 ± 8.0	547.02 mg/d anthocyanins for 7 d	Cycling trial	Work performed by cycling	NS
[83]	Montmorency tart cherry supplement (Cherry Active®)	Dextrose capsules	Db RPCCT	0 + 8	19.7 ± 1.6	256.8 mg/d anthocyanins for 7 d	Cycling time trial	Time trial completion time	Improvement
[84]	Montmorency tart cherry (Cherry PURE®)	Rice flour capsules	Db RPCT	9 + 18	21.8 ± 3.9	66 mg/d anthocyanins for 10 d	Running (half-marathon)	Finish time	Improvement

Table 2. *Cont.*

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
[86]	Integral purple grape juice	Isoenergetic carbohydrate-based beverage	Db RPCT	6 + 22	39.8 ± 8.5	10 mL/kg/d containing 52.6 mg/L anthocyanins for 28 d	Treadmill running	Time to exhaustion	Improvement
[87]	Blueberry powder	Carbohydrate and fiber-matched placebo powder	Db RPCT	0 + 59	39.0 ± 2.0	345 mg/d anthocyanins for 2 wks	Cycling	Time trial	NS
Ellagitannins									
[88]	Pomegranates	Carbohydrate-matched placebo drink	Db RPCCT	0 + 12	26.8 ± 5.0	171.9 mg/d ellagitannins for 7 d	Cycling in the heat	Time trial	NS
[89]	Pomegranate extract	Pure stevia extract powder	Db RPCCT	2 + 6	37 ± 11	15 mg/kg/d containing 11.46 mg/kg/d ellagitannins for 8 d	Cycling time trial	Average power outputs and energy expenditure	NS
[90]	Pomegranate extract (POMANOX® P30)	Maltodextrin capsules	Db RPCCT	0 + 24	34.9 ± 10	225 mg/d punicalagins for 15 d	Cycling trial	Time to exhaustion	Improvement
[91]	Pomegranate juice (Oleofarm®)	Water, sugar, and grenadine	Db RPCT	0 + 19	20.8 ± 0.86(SUP) 20.9 ± 0.95(PL)	50 mL/d juice containing 220 mg/100 g polyphenols for 2 months	Rowing ergonometer	Time to complete 2000 m	NS

Table 2. *Cont.*

Family Reference	Flavonoid source	Control Groups	Study Design	Number of Participants (Female + Male)	Mean Age of Participants (Years)	Dosage	Exercise	Performance Variable	Effect
Isoflavones									
[92]	Peptides, taurine, *Pueraria* isoflavone, and ginseng saponin complex	Starch and lactose	Db RPCCT	0 + 14	21.6 ± 0.7	180 mg of isoflavone for 15 d	Cycling	Time to exhaustion	Improvement
Flavones									
[93]	Peanut husk extract	Microcrystal-line cellulose capsules containing maltodextrin	Db RPCCT	0 + 12	21.3 ± 2.1	50 or 100 mg/d luteolin for 15 d	Cycling trial	Peak power	Improvement

Tb = triple-blind, Db = double-blind, Sb = single-blind, RCT = randomized controlled trial, RPCT = randomized placebo-controlled trial, RPCCT = randomized placebo-controlled crossover trial. LD = low-dose, HD = high-dose, PL= placebo, SUP = flavonoid-supplemented, d = day, wk = week, and NS = nonsignificant effect.

3.3. Risk of Bias within Studies

The risk of bias (selection bias, performance bias, detection bias, attrition bias, reporting bias, and other sources of bias) was established within the 54 articles considered (Figure 4).

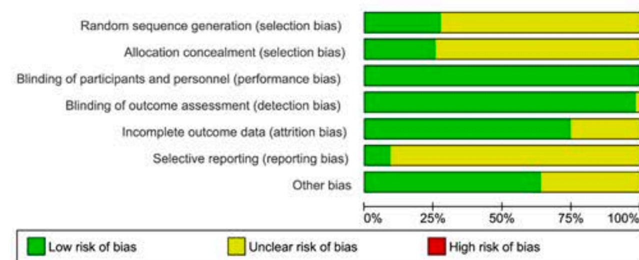

Figure 4. Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

In performance bias, detection bias, attrition bias, and other bias, over 50% of articles were assessed as "low-risk". Performance bias and detection bias were assessed as "low-risk" in almost all the articles, because they assure the blinding of participants, investigators, and outcome assessment during the intervention. In the included articles, incomplete outcome data was not considered as a potential risk of bias.

The selection bias and the reporting bias scarcely went over 25%. The selection bias domain was assessed as an "unclear risk" in most of the articles, because the authors did not specify how a random sequence was generated, and, consequently, it provided an inappropriate way to evaluate the allocation concealment. In most of the studies, the reporting bias domain was assessed as an "unclear risk" due to a lack of an available protocols or not having information enough to assess this domain.

Another bias considered was if studies did not include a detailed explanation about the participation of sponsors in the intervention and the subsequent results.

3.4. Association between Flavonoid Intake, Performance, Immune System, and Inflammatory Biomarkers

Besides the influence of the flavonoid consumption in exercise performances, we aimed to establish the relationship of these effects with the immune system functionality of the participants. From the 54 articles selected in the systematic review, only 18 articles included measures of the immune system (Table 3). From these articles, six articles referred to the quercetin administration [50,52,56,59–61], with two of them focused on the same population [52,59]; six other articles applied extracts with flavanols [26,65,67,85,97,98], with two of them focused on the same clinical trial [97,98]; five articles used extracts enriched in anthocyanins [77,82,84,86,87], and one referred to a pomegranate juice with ellagitannins [91]. Most of these studies focused on the inflammatory response associated with the exercise, which was quantified by means of plasma C-reactive protein (CRP) and inflammatory and anti-inflammatory cytokines, mainly the myokine IL-6. From the 18 selected articles, only two quantified a biomarker of acquired immunity, such as salivary immunoglobulin A (IgA) [52,61]; moreover, two articles focused on the incidence of URTI [52,67], and one determined the ex vivo antibacterial and antiviral activities [98].

Firstly, with regards to those studies focused in quercetin supplementation, Nieman et al. [59] determined the levels of plasma inflammatory cytokines and chemokines such as IL-6, IL-1ra, IL-8, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor (TNF) α, and the anti-inflammatory IL-10, as well as the leukocyte and muscle gene expression of

IL-1ra, IL-8, and IL-10 in subjects submitted to three three-hour cycling bouts. The cytokine levels increased after exercise. Quercetin supplementation (1000 mg/day for three weeks) did not improve the physical performance and was only able to reduce the leukocyte gene expression of the inflammatory IL-8 chemokine and the anti-inflammatory IL-10 cytokine [59]. Nevertheless, the immune function was also studied in the same subjects by means of the NK cell activity, proliferative activity, polymorphonuclear oxidative-burst activity, and the levels of salivary IgA. None of these immune function markers were affected by the quercetin administration, but there was a lower incidence of URTI in the two-week postexercise period in supplemented cyclists compared to the placebo group [52]. In another study, Nieman et al. [60] measured inflammation by means of CRP, IL-1ra, IL-6, IL-8, IL-10, MCP-1, TNF-α, granulocyte colony-stimulating factor (G-CSF), and macrophage inflammatory protein (MIP-1β) and the leukocyte gene expression of some cytokines in ultramarathoners competing in the 160-km Western States Endurance Run who received 1000 mg/day of quercetin for three weeks before the race. In this case, quercetin was also unable to modify the physical performance or attenuate the CRP-increased levels or the increases in the plasma cytokines, and it also failed to attenuate the muscle damage. This was suggested to be due to the extreme exertion induced by running a 160-km trail race.

Nieman et al. [61] also studied the effect of two weeks of 1000 mg/day of quercetin administered with vitamin C (1000 mg/day) together or not with EGCG (120 mg), isoquercetin (400 mg), and PUFA (400 mg). As commented on above, no effects on the cycling performance by any supplement were found, but there was a greater granulocyte oxidative burst at the baseline and a decrease in plasma CRP, IL-6, and IL-10 immediately after the exercise bout [61]. The blood leukocyte count and salivary IgA were also established in these athletes. The blood leukocyte number tended to be lower after exercise in the quercetin and quercetin plus ECGC groups compared to the placebo, with significant lower levels 14 h after exercise, but no significant differences were found due to exercise or supplementation in the ratio of salivary IgA to protein [61].

On the other hand, it was reported that one week of quercetin-3-glucoside supplement was not able to prevent the increase in plasma IL-6 levels associated with repeated sprints of team sports-trained athletes or increase in their performances [56]. Similarly, quercetin did not modify the plasma IL-6 and CRP levels and did not prevent the strength loss, muscle soreness, reduced arm angle, CK elevation, and arm swelling in individuals performing two separate sessions of 24 eccentric contractions of elbow flexors [50].

Some studies focused on the extracts containing flavanols, anthocyanins, or ellagitannins, and physical performances have also shown the effect of supplements on the immune system (Table 3). A supplement of green tea extract containing 159 mg/day of catechins (flavanols) was unable to modify the cycling performance, and in comparison with the placebo group, there was also no difference in the inflammatory IL-6 cytokine, but there was a decrease in the plasma CRP levels [85]. Moreover, flavanols from a complex of blueberry–green tea–polyphenol soy protein (1001 mg/day containing 2136 mg of gallic acid equivalents for 17 days) did not improve the running distance of trained long-distance runners or prevent the biomarkers of inflammation such as the white blood cell count, plasma CRP, IL-6, and MCP-1 levels [97]. Interestingly, the immune system function of these athletes was established by means of ex vivo studies about antibacterial and antiviral activities [98]. No effect on the growth of Gram-negative and Gram-positive bacteria was found; however, the blueberry–green tea–polyphenol soy protein complex showed, by unknown mechanisms, a protective effect on virus infectivity [98]. These results suggest the potential of this flavanol mixture in the protection against viruses that often occur following intensive exercise.

Table 3. Summary of the included studies assessing the association between flavonoid intake, exercise performance, and immune status biomarkers in humans.

Reference	Flavonoid	Dosage	Exercise	Effect on Performance	Measurement	Outcome
Quercetin						
[59]	quercetin + Tang powder	1000 mg/d for 3 wks	Three 3-h cycling bouts	NS	• plasma inflammatory cytokines (IL-6, IL-10, IL-1ra, IL-8, MCP-1, TNF-α) • leukocyte mRNA • muscle mRNA	• = plasma inflammatory cytokines • ↓ leukocyte gene expression of IL-8 and IL-10
[52]	quercetin + Tang powder	1000 mg/d for 3 wks	Three 3-h cycling bouts	NS	• NK cell activity • proliferative activity • PMN oxidative-burst activity • salivary IgA • incidence of URTI	• = NK cell activity • = proliferative activity • = PMN oxidative-burst activity • = IgA • ↓ incidence of URTI
[60]	quercetin + vit C + niacin	1000 mg/d quercetin + 1000 mg/d vit C + 80 mg/d niacin for 3 wks	160-km Western States Endurance Run	NS	• CRP • Plasma inflammatory cytokines (IL-1Ra, IL-6, IL-8, IL-10, G-CSF, MCP-1, MIP-1β, TNF-α, MIF-1) • leukocyte gene expression of some cytokines	• = CRP • = plasma inflammatory cytokines • = leukocyte gene expression
[56]	quercetin-3-glucoside + 6% carbohydrate sports drink	1000 mg/d for 1 wk	Running repeated sprints	NS	• plasma IL-6	• = plasma IL-6
[61]	Quercetin + isoquercetin + EGCG	1000 mg quercetin + 120 mg EGCG + 400 mg/d isoquercetin for 14 d	Cycling	NS	• plasma CRP • plasma IL-6 and IL-10 • blood leukocyte counts • salivary IgA	• = plasma CRP • = plasma IL-6 and IL-10 • ↓ blood leukocyte counts • = salivary IgA
[50]	Quercetin + vit C + tocopherols	1000 mg/d quercetin + 20 mg/d vit C + 14 mg/d tocopherols for 1 wk	Eccentric contractions of the elbow flexors	NS	• plasma IL-6 • plasma CRP	• = IL-6 • = CRP

Table 3. *Cont.*

Reference	Flavonoid	Dosage	Exercise	Effect on Performance	Measurement	Outcome
Extracts with flavanols						
[85]	Green tea extract	159 mg/d catechins for 3 wks	Cycling	NS	• plasma IL-6 • plasma CRP	• = IL-6 • ↓ CRP
[97]	Blueberry–green tea–polyphenol soy protein complex	1001 mg/d flavanols for 17 d	Running in a treadmill for 2.5 h	NS	• WBC count • serum CRP • plasma IL-6, MCP-1	• = WBC • = CRP • = plasma IL-6, MCP-1
[98]	Blueberry–green tea–polyphenol soy protein complex	1001 mg/d flavanols for 17 d	Running in a treadmill for 2.5 h	NS	• ex vivo antibacterial activity • ex vivo antiviral activity	• = ex vivo antibacterial activity • ↑ ex vivo antiviral activity
[65]	Flavanol-rich lychee fruit extract	100 mg/d flavanols for 2 months	Running training, combining low, medium, and high intensities	NS	• Total and differential WBC counts • CRP • serum inflammatory (IL-6) and • anti-inflammatory cytokines (IL-10, TFG-β1, TFG-β2)	• ↓ WBC counts, = neutrophil and lymphocyte counts • = CRP • = absolute IL-6, IL-10, TFG-β1, TFG-β2 • ↓ % IL-6 and TFG-β1 from pre-training to mid-training period
[67]	Nonalcoholic beer	1.0–1.5 L/d with 47 mg/L catechin and 33 mg/L procyanidins for 3 wks	Munich marathon race	NS	• IL-6 • CRP • total blood leukocyte counts • incidence of URTI	• ↓ IL-6 • ↓ CRP • ↓ WBC • ↓ URTI incidence
[26]	Dark chocolate	197.4 mg flavanols for 2 wks	Incremental cycling	NS	• inflammatory and anti- • inflammatory cytokines • WBC, neutrophil counts	• = IL-6, IL-1ra, IL-10 • = WBC, neutrophil counts

Table 3. *Cont.*

Reference	Flavonoid	Dosage	Exercise	Effect on Performance	Measurement	Outcome
Extracts with anthocyanins						
[77]	New Zealand blackcurrant	210 mg/d anthocyanins for 7 d	Chichester half-marathon	NS	• urine IL-6	• = urine IL-6
[82]	Montmorency tart cherry concentrate	547.02 mg/d anthocyanins for 7 d	Cycling trial	NS	• hs-CRP blood • inflammatory cytokines	• ↓ hs-CRP • ↓ IL-6 • = IL-1β, IL-8, TNFα
[84]	Montmorency tart cherry	66 mg/d anthocyanins for 10 d	Running (half-marathon)	Improvement	• serum inflammatory cytokines (TNF α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-12p70) • serum anti-inflammatory cytokines (IL-4, IL-5, IL-7, IL-10, IL-13) • total and differential WBC • GM-CSF	• ↓ IL-6, ↓ IL-2, = remaining cytokines • ↓ IL-13, = remaining cytokines • = WBC • = GM-CSF.
[86]	Integral purple grape juice	10 mL/kg/d containing 52.6 mg/L anthocyanins for 28 d	Treadmill running	Improvement	• serum AGP • hs-CRP • WBC	• ↓ AGP • = hs-CRP • = WBC
[87]	Blueberry powder	345 mg/d anthocyanins for 2 wks	Cycling	NS	• Inflammatory cytokines • Oxylipins	• = IL-6, IL-1ra • ↓ some oxylipins
Extracts with ellagitannins						
[91]	Pomegranate juice	50 mL/d juice containing 220 mg/100 g polyphenols for 2 months	Rowing ergonometer	NS	• Serum inflammatory cytokines	• = IL-6, IL-1ra

↓ = decrease, ↑ = increase, AGP = α-1-acid glycoprotein, CRP = plasma C-reactive protein, GM-CSF = granulocyte colony-stimulating factor, hs= high-sensitivity, IFN = interferon, IgA = immunoglobulin A, IL = interleukin, MCP-1 = monocyte chemoattractant protein-1, NK = natural killer, PMN = polymorphonuclear leukocytes, TGF = transforming growth factor, TNF = tumor necrosis factor, URTI = upper-respiratory tract infections, vit = vitamin, WBC = white blood cells.

Another extract enriched in flavanols, such as a lychee fruit extract with no effect on running performance in comparison with placebo group, showed interesting findings in immune-related biomarkers. Participants with supplement exhibited a lower white blood cell count increase after the training period, although no changes in neutrophil or lymphocyte counts were observed throughout the training period [65]. CRP levels and absolute serum IL-6 concentrations were not modified by the supplementation. However, the percent decrease in IL-6 from the pre-training to mid-training period was significantly smaller in the participants taking lychee extract. Similarly, the levels of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TFG) β1 and β2 showed that, although the absolute concentrations were not significantly modified, the percentage increase from pre-training to post-training was significantly greater or tended to be higher for TFG-β1 and TFG-β2 concentrations, respectively, between the flavanol-supplemented and placebo groups [65]. The preventive effect of flavanols in some changes in the immune system is reinforced by the study by Scherr et al. [67]. In this case, a nonalcoholic beer providing about 47 mg/day of catechin and 33 mg/day of procyanidins for three weeks in runners of the Munich marathon prevented the increase in IL-6, CRP, and total blood leukocyte counts. Interestingly, this study demonstrated that the incidence of URTI was lower in the nonalcoholic-runner group in the two weeks after marathon competition, a period in which the supplement was also given [67]. On the other hand, Allgrove et al. [26] also assessed the effect of a two-week flavanol intake (197.7 mg/day) by means of dark chocolate in cycling. Dark chocolate did not improve the cycling performance but decreased the plasma levels of the oxidative stress biomarkers without affecting the plasma concentration of cytokines, such as IL-6, IL-10, and IL-1ra, or the blood counts of leukocytes and neutrophils after prolonged exercise [26].

As reported above, the effect of supplements containing anthocyanins has been studied in exercise performances and, in some cases, in biomarkers of the immune system. In a recent study performed in Chichester, half-marathon runners received 210 mg/day of anthocyanins from New Zealand blackcurrants; however, the flavonoids did not modify the finish time, and there was also no change in the urine IL-6 concentration [77]. Another study with anthocyanins, but from Montmorency tart cherries (547.02 mg/day anthocyanins for seven d) in well-trained cyclists, analyzed the levels of blood inflammatory cytokines and high-sensitivity CRP (hsCRP) after a stochastic road cycling trial for three consecutive days [82]. Whereas nonsignificant improvement was found for the cycling work performed, the increase in plasma IL-6 and hsCRP was attenuated by the Montmorency tart cherry concentrate, which also showed a significant effect on the oxidative stress markers. However, no influence of anthocyanins was reported for the increased levels of IL-1β, IL-8, and TNF-α [82].

Levers et al. [84] also analyzed the effect of Montmorency tart cherries but derived from tart cherry skins obtained after juicing, with a lower content of anthocyanins (66 mg/day) and for a longer period (10 days). Endurance-trained runners or triathletes racing in a half-marathon taking such a supplement produced a faster race and also experienced attenuated markers of muscle damage, oxidative stress, inflammation, and perceptions of muscle soreness than the placebo group. With regards to the inflammatory response, the serum levels of inflammatory cytokines (TNF-α, interferonγ, IL-1β, IL-2, IL-6, IL-8, and IL-12p70) and anti-inflammatory cytokines (IL-4, IL-5, IL-7, IL-10, and IL-13) were measured. The serum IL-6 concentration was attenuated by the extract in the measures 60 min post-run, whereas IL-2 and IL-13 were significantly decreased by anthocyanins at 60 min, 24 h, and 48 h of running. On the other hand, the analysis of the total and differential white blood cell counts and the granulocyte-macrophage colony-stimulating factor (GM-CSF) showed no significant changes between the placebo and supplemented groups.

Similarly to the study by Levers et al. [84], Toscano et al. [86] applied anthocyanins in recreational runners and observed an improvement in the time-to-exhaustion. In this case, the supplement derived from purple grape juice from Brazil contained 52.6 mg/L

of anthocyanins and was administered at 10 mL/kg/day for 28 days. The analysis of serum α-1-acid glycoprotein (AGP) and hs-CRP concentrations and the blood total and differential white cell counts showed that the AGP levels decreased by grape juice at 14 and 28 days, with nonsignificant effects on the hs-CRP or on white cell counts. In parallel, there was an increase in the antioxidant activity by the extract [86].

More recently, as reported by Nieman et al. [87], anthocyanins from blueberries (345 mg/day for two weeks), together or not, with acute banana intake before a 75-km cycling trial did not improve the performance or plasma IL-6 and IL-1ra concentrations, although the banana consumption decreased the IL-1ra levels. This study also focused on other plasma inflammatory biomarkers such as oxylipins generated during stressful exercise from the n-6 and n-3 PUFA metabolism by the cyclooxygenase, lipoxygenase, and cytochrome P450 pathways [104]. Some of these biomarkers (those derived from the cytochrome P450 pathway) decreased due to both blueberry and/or banana ingestion.

Finally, another family of flavonoids, ellagitannins, has been studied both in exercise performance and the immune system. As reported before, Urbaniak et al. [91] used a supplement of pomegranate juice (two months) rich in ellagitannins in rowing on an ergonometer. The authors observed a higher antioxidant capacity after pomegranate fruit juice ingestion, although there was no improvement on the time to complete two km of rowing. The serum IL-6 concentration analysis also showed no changes in this inflammatory cytokine between the placebo and supplemented groups.

In summary, considering the clinical trials that applied flavonoids and quantified physical performance and immune system status, only two administering extracts with anthocyanins [84,86] showed an improvement in the exercise performance and measured some immune markers. Both these studies showed that anthocyanins lowered the inflammatory response after the quantification of IL-6 [84] or α-1-acid glycoprotein [86] but did not modify other inflammatory cytokines, CRP, or the white blood cell counts. In comparison with the other three studies using anthocyanins and determining the immune functions, the successful studies used lower the flavonoid intake (52.6–66 mg/day), but it was administered for a longer period (10–28 day) in runners, whereas the other three studies focused on cyclists or runners taking more than 200 mg/day of anthocyanins for 7–14 days (Table 3). On the other hand, extracts with flavanols [67,98] or quercetin alone [52] that did not improve the physical performance were able to decrease the incidence of URTI in athletes after intense exercise (in one case, a marathon race) or increase the ex vivo antiviral activity.

4. Discussion

The aim of the current review was to systematically assess the available evidence published in the last 15 years about the potential benefit of flavonoids on human sport performance when consuming them for at least seven days. To our knowledge, this is the first systematic review of the effect of flavonoids, the most consumed polyphenol class [3], on human exercise performances. From 2005 to 2020, 54 articles were selected according to the established criteria (healthy adult people, randomized, controlled trial, either single- or double-blind study designs, interventions lasting for at least seven days, and physical exercise performance objectively quantified).

The overall proportion of the reviewed articles that clearly showed an improvement in athletic performance due to flavonoid supplementation was 37% (representing the 27.5% of the participants included in this review, 30.8% women and 27.1% men), which does not allow the conclusion to be made with certainty that flavonoid consumption provides ergogenic effects. Nevertheless, when considering the different flavonoid subclasses separately, anthocyanins seem to exert a greater ergogenic effect, because the proportion of successful results after their consumption increases to 54%.

Considering a pure flavonoid intake, nearly all the studies focused on quercetin. Of these, only 25% (3/12) demonstrated that quercetin improved exercise performances [48,55,58]. Nieman et al. [55] and Davis et al. [48] hypothesized that the

outcome could depend on the fitness level of the participants and suggested that the performance is more likely to be improved due to quercetin in untrained subjects, since trained participants have already reached a higher threshold regarding the antioxidant and mitochondrial capacities [105]. In fact, the three studies that confirmed quercetin's ergogenic properties were carried out in untrained moderately active people [48,55,58], whereas the studies using trained male cyclists [52,59,63], endurance runners [60], student athletes [49,51], team sports-trained athletes [56], and military trained participants [57,62] found no beneficial effects on sports performances. However, Cureton et al. [47] and O'Fallon et al. [50] also studied the potential effects of quercetin on untrained subjects and found no changes, although the first of these authors reported a nonstatistically significant 2.7% increase in VO_{2max} following the supplementation that was not observed in the placebo condition. The observation of nonergogenic effects by these authors seems not to be related to an inadequate dose/duration of the supplementation, since Davis et al. [48] reported an improvement after just one week of 1000-mg/day quercetin intake. On the other hand, the short half-life of quercetin (3.5 h) is known [106], and in two studies reporting an improvement in performance, quercetin was consumed one h [48] and two h [59] before the exercise test, which could be important for enhancing the sports ability. Additionally, it could be hypothesized that the improvement could be due to the interaction of quercetin with other ingredients that could influence its bioavailability. Nevertheless, in the studies reporting an improvement in performance, quercetin was administered both alone [58] or in sugar-free sports beverages provided by Tang (Kraft Foods) [48] or Coca-Cola [55]. Moreover, in the unsuccessful study by Cureton et al. [47], quercetin was also consumed in a sports hydration beverage prepared by the Coca-Cola Company, and in the study by O'Fallon et al. [50], participants received quercetin through First Strike nutrition bars (Natick Soldier Center), which also contained vitamin C and tocopherols. In fact, most of the studies provided quercetin in combination with other food components, such as vitamin C, folate, PUFA such as EPA and DHA, and even other flavonoids that could influence the quercetin absorption. Although there is increasing evidence that these combinations may enhance quercetin's bioavailability and bioactive properties, as observed in both the preclinical [107,108] and clinical studies [61,109], both successful and unsuccessful studies reported the quercetin levels in the blood [47,52,55–57,59,60,62]. Nevertheless, it is known that quercetin bioavailability exhibits a high interindividual variation, which can be due, among other factors, to genetic polymorphisms, dietary adaptation, the composition of gut microbiota, and other subject characteristics such as the body mass index [110]; therefore, the controversial outcomes reported could be due to intrinsic individual factors beyond its dosage.

Several mechanisms could explain the potential ergogenic effects of quercetin. In preclinical studies performed in sedentary mice, quercetin has been shown to stimulate mitochondrial biogenesis throughout enhancing the muscle and brain mRNA expression of sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α), as well as increasing the mitochondrial DNA (mtDNA) and cytochrome C concentrations in the muscles and brain, leading to an increase in the skeletal muscle oxidative capacity and running performances [111]. In addition, an increase in mitochondrial biogenesis and muscle oxidative capacity may alter the substrate utilization during long bouts of endurance exercise by increasing the oxidation of fat and sparing muscle glucose and glycogen reserves [48]. Nevertheless, previous studies have assessed the effects of quercetin supplementation on fuel utilization in both untrained [47] and well-trained [112] cyclists, and no clear effects were found. The antioxidant properties of quercetin could also explain the potential ergogenic effect by reducing the muscle damage and soreness, as well as attenuating the decline observed in the neuromuscular performance due to the increased ROS production during exercise [58]. Moreover, in vitro studies suggest that quercetin may be an adenosine A_1-receptor antagonist [113] and, hence, may exert analgesic effects that could decrease the effort perception or muscle aches and pain during exercise.

Regarding other pure flavonoid supplementation strategies, Overdevest et al. [54] found a 5% increase in the cycling power output after four weeks of 500 mg/day of hesperidin supplementation, a comparable improvement with that observed after creatine supplementation [114]. These positive results are even more relevant when considering that the participants were well-trained athletes with an average exercise time per week of 9.6 h. The authors suggested that this performance improvement could be due to hesperidin's antioxidant properties, as well as other intracellular effects at the mitochondrial level, in a similar manner as previously explained for quercetin [54]. In agreement with these findings, Martínez-Noguera et al. [115] reported an increase in average power, maximum speed, and total energy during a repeated cycling sprint test only five h after the acute intake of 500 mg of 2S-Hesperidin (Cardiose®). Moreover, the preclinical research also supported the ergogenic effect of hesperidin [21] and its positive effects against exercise-induced oxidative stress [116,117]. Further studies should be carried out to confirm these promising results and establish the optimal intake duration for achieving these effects.

Flavanols from cocoa, administered as cocoa flavanol capsules [68], cocoa powder [71], chocolate [26,69,70], or as a capsule containing only (-)-epicatechin [53], were also assessed for improving performances. None of (-)-epicatechin [53], dark chocolate [26,69], or cocoa flavanol [70,71] consumption resulted in a better performance in recreationally active people [26,53], trained cyclists [68,69], cross-country athletes [71], and elite rugby players [70]. However, the cocoa flavanol intake may be a good strategy to counteract exercise-induced oxidative stress [26,68] and to confer some metabolic benefits [69,71]. Decroix et al. [19] reviewed the benefits of cocoa flavanol supplementation in sports and concluded that it may attenuate exercise-induced oxidative stress, improve muscular mitochondrial efficiency and VO_{2max} in untrained subjects, and positively alter fat and carbohydrate utilization during exercise without inducing changes in the exercise performance.

Regarding other flavanol-enriched extracts, eight articles assessed the impact of green tea extract supplementation on exercise performances [28,64,85,94–99], and 25% (two out of eight) found successful results in recreationally active males [94,95]. Previous studies in mice have reported an improved endurance performance following tea catechin intake [118,119], as well as an enhanced fatty acid oxidation in the liver and skeletal muscles [119]. Moreover, a synergic effect between tea catechins and other tea components such as caffeine and theanine have been reported with regards to lipid metabolism alterations [120] and could also be found in exercise-induced changes. On the other hand, the potential ergogenic effect of lychee fruit extract was studied by Kang et al. [66] and Nishizawa et al. [65]. Where a 200-mg/day intake of oligomerized lychee fruit extract for one month resulted in successfully enhancing the submaximal running time and increasing the anaerobic threshold in regularly exercising male participants [66], the intake of 100 mg/day of flavanol-rich lychee fruit extract for two months did not alter the five-km running time performances [65]. However, Nishizawa et al. [65] observed anti-inflammatory effects in young athletes after two months of 100 mg/day of lychee supplementation, as has been previously suggested in animal models [121,122]. Kang et al. [66] also studied the effects of 30 days of oligomerized lychee fruit extract supplementation with a mixture of vitamin C (800 mg) and vitamin E (320 IU) and reported an attenuation in VO_{2max}. Further research may confirm these interesting findings and establish the optimal dosage of lychee fruit extract for obtaining an improvement in performances.

Another three studies assessed the impact of other flavanol-enriched extracts on exercise performances [67,72,74], and two of them reported improvements [72,74]. In particular, apple [74] and carob [72] extracts containing proanthocyanidins, among other flavonoids, successfully improved the two-hour cycling trial performance and distance covered in response to a Yo-Yo intermittent recovery test, respectively. Ataka et al. [74] also reported no impact on the two-hour cycling trial performance following a supplementation with the antioxidant ascorbic acid (1000 mg/day for eight days), suggesting that the mechanisms through which apple procyanidins intake could improve exercise performances may not be exclusively related to their antioxidant properties. These results, together with those

reported by Kang et al. [66], are in line with preclinical [123–125] and human studies [125] suggesting that the intake of great amounts of antioxidant vitamins could attenuate or even inhibit the improvements of the endurance capacity through the blockage of exercise-induced ROS production. With regards to other tannins, four studies [88–91] assessed the potential ergogenic effects of ellagitannins from pomegranates, especially punicalagins. Positive results were only found in trained cyclists [90]. This improvement in their performances may be due to ellagitannins' ability to enhance the blood flow and vessel diameter. Another study [126], which was excluded from the current review for not matching the intake duration inclusion criteria, reported an improvement in time to exhaustion at 90% in highly active participants following acute pomegranate extract supplementation only 30 min before exercising. However, these acute effects of pomegranates on exercise performances could be related with their high nitrate contents [127]. Overall, 28% (5/18) of the studies reviewed reported flavanols' ergogenic effects [66,72,74,94,95], the most promising sources being apple, green tea, lychee fruit, and carob extracts. In particular, 37.5% (3/8) of the studies assessing cycling performances observed an improvement following flavanol intervention, whereas the proportion of studies finding flavanol-induced enhancement in running performances was 25% (two out of eight). These findings, together with the several health benefits that have been attributed to dietary flavanols [128,129], deserve further investigation.

Anthocyanin supplementation seems to be the most promising strategy for improving exercise performances. Thirteen of the studies reviewed assessed the effects of this flavonoid family, and 54% (7/13) found promising results for athletes. The sources of anthocyanins include blackcurrants, Montmorency tart cherries, integral purple grape juice, and blueberries.

Eight studies focused on New Zealand blackcurrant (NZBC) supplementation, containing mainly the anthocyanin delphinidin-3-rutinoside, and one-half of them reported an improvement in the performances of trained participants. It is important to highlight that these ergogenic effects were observed in either men performing the cycling exercise for 8–10 h/week [73], recreationally active men with experience in sports involving high-intensity intermittent exercise who were familiarized with treadmill running [76], and male climbers with more than three years of regular climbing experience [81]. In addition, Braakhuis et al. [75] found controversial results in trained female runners; where the average and faster (+one standard deviation of the mean speed) runners' performances worsened following blackcurrant intakes, very fast runners (+two standard deviation of the mean speed) improved their running performances following the supplementation. They hypothesized that these results come from a synergic effect between the blackcurrant supplementation and a greater training load. In addition, Fryer et al. [79,80] found better muscle oxygenation during repeated forearm muscle contractions in intermediate, advanced, and elite climbers following NZBC supplementation that was not accompanied by an improvement in climbing performances. Nevertheless, assessing the performance changes was not the primary goal of their studies, and, as the authors stated, there were some limitations in the manner they evaluated them. Where, in advanced and elite climbers [79], the workload at 60% applied in the maximal volitional contraction (MVC) could not be intensive enough, in intermediate-level climbers [80], the coefficient of variation for the MVC was quite high (4.37%), and this could denote a lack of ability to objectively evaluate their forearm performance. Similarly, Cook et al. [78] assessed performances during submaximal isometric contractions (30% MVC), and Costello et al. [77] mainly aimed to assess changes in the recovery from half-marathon-induced muscle damage, finding no changes in the recovery of muscle function, muscle soreness, and fatigue and no differences in the half-marathon finish time.

The potential mechanisms through which blackcurrant anthocyanins could improve performances are quite unclear but seem to be related to the positive effects on endothelial function [73]. Blackcurrant anthocyanins have the potential to increase nitric oxide by endothelial cells and decrease the breakdown by nitric oxide free radicals, probably leading

to enhanced skeletal muscle blood flow and contractile efficiency [73,130,131]. Overall, in agreement with Braakhuis et al., who recently reviewed the effect of NZBC on sport performances [17], we can conclude that a seven-day NZBC intake providing a daily dose of 105–210 mg/day of anthocyanins and including a final dose one to two h before exercising may result in a significant improvement in athletic performance. However, since the peak levels of anthocyanin in the bloodstream seem to appear two h post-consumption [132], further studies are recommended to elucidate whether the ergogenic effects observed reflected the seven-d intake or the acute intake.

In recent years, Montmorency tart cherries have gained increasing attention, especially with regards to their potential clinical applications in exercise-induced muscle damage recovery, inflammation, and oxidative stress [133]. From the three reviewed articles assessing their potential ergogenic effects [82–84], 66.6% (2/3) found successful results in endurance-trained runners or triathletes [84] and trained-male cyclists [83]. Bell's study [82] focused on assessing the effects of Montmorency tart cherry concentrate on oxidative stress, inflammation, and muscle damage biomarkers, as well as on the expected decrease in performance across three consecutive days of cycle racing; they reported the effects on the cycle work performed as a secondary outcome, which could influence the non-observation of significant changes. As the authors suggested [82], further research should focus on functional performance assessments. The mechanisms through which tart cherries may improve exercise performances seem to be related with their high contents in cyanidin-3-glucoside. This anthocyanin has been demonstrated to enhance mitochondrial biogenesis in both mice [134] and a human hepatocyte cell line [135] through upregulating skeletal muscle PGC-1α, leading to an activation of lactate metabolism [134] and, finally, enhancing the skeletal muscle oxidative capacity and improving the endurance performance in a similar manner as previously suggested for quercetin [111]. Moreover, the upregulation of PGC-1α induced by the cyanidin-3-glucoside intake increases the expression of vascular endothelial growth factor α (VEGFα), improving the muscle blood supply and oxygen availability and, hence, explaining a potential improvement in exercise performances [134]. Overall, both blackcurrant and tart cherry extract intakes seem to be promising strategies to enhance exercise performances. However, the predominant anthocyanins in these two extracts are different, so the mechanisms underlying the potential ergogenic effects may differ and deserve further investigation.

Grape juice supplementation was also demonstrated to be effective in increasing performances in recreational runners who carried out four to five training sessions per week (78-min average training time/session) [86]. In addition, a recent study from the same authors concluded that just a single dose of grape juice two h before exercise is able to enhance runner performances [136]. Although the mechanisms underlying these effects remain unknown, the authors hypothesized that the anti-inflammatory, antioxidant [86,136,137], and vasodilator properties [138] of grape juice could lead to a better recovery between daily training sessions and, hence, an overall better exercise performance.

Apart from the most common studies on flavanols and anthocyanins, two studies reported the positive effects of a 15-day supplementation with isoflavones and flavones on cycling performances [92,93]. In particular, an encapsulated blend containing soybean peptides, taurine, *Purearia* isoflavone, and ginseng saponin increased the time to exhaustion in a cycling test, probably through enhancing the lipid utilization as an energy substrate while sparing the glycogen reserves, since increased nonesterified fatty acids blood levels during exercise were found [92]. On the other hand, a 15-day supplementation with a combination of peanut husk extract containing the flavone luteolin and mango leaf extract resulted in better sprint performances, probably through the facilitation of muscle oxygen extraction, the reduction of oxygen consumption during sprints, and an increase in the lactate blood levels [93]. It is worth noting that this study included two different doses, both showing similar effects, indicating that no dose response was achieved and suggesting that the effect of flavonoids may be limited, and no higher doses would for certain provide more successful effects.

Besides the influence of flavonoid consumption in exercise performances, we aimed to find the relationship between these effects with the immune system status of the participants. It is well-established that moderate physical activity enhances immune functions [32–34], but intense exercise induces inflammation, alters phagocytic and cytotoxic functions, decreases mucosal IgA, and increases the susceptibility to infections, especially URTI [40,42]. In recent years, flavonoids have shown immunomodulatory properties in both animal and human studies [139–142]. In fact, a recent review [142] demonstrated that flavonoid consumption decreases the URTI incidence by 33% compared to the control, and two studies [143,144] found significant reductions in URTI symptoms with cranberry beverages or garlic extract. Therefore, beyond the protective effects of flavonoids in the overproduction of ROS associated with intense exercise [25], flavonoids could counteract the immune changes and, eventually, protect against the increase in URTI incidences that often accompany intense physical exercise.

From the 54 selected articles considering the effect of flavonoid intake on exercise performance, only 18 articles referred to physical performances and the immune status [26,50,52,56,59–61,65,67,77,82,84–87,91,97,98], and only two [84,86] of these articles coincided with better performances induced by flavonoid consumption. Unfortunately, most of the 20 articles demonstrating the beneficial influence of flavonoid in exercise performances did not focus on the immune status. Both successful studies [84,86] showed that anthocyanins were able to increase the performances and, at the same time, lowered the inflammatory response by decreasing IL-6 [84] or α-1-acid glycoprotein [86], although they did not modify other inflammatory cytokines, CRP, or the white blood cell counts. Therefore, it is difficult to associate the improvement in performances by flavonoids with a biomarker of the immune system.

Regardless of their effect on exercise performances, most of the articles that considered the immune system focused on the inflammatory response, and actually, only three evaluated the immune functionality by means of the incidence of URTIs and ex vivo antiviral and antibacterial activities [52,67,98]. Interestingly, two studies demonstrated that the intake of quercetin (1 g/day for three weeks) [52] or a nonalcoholic beer enriched in catechins (for three weeks) [67] was able to decrease the incidence of URTIs in cyclists and marathon runners, respectively. Nevertheless, these studies did not find a better performance with flavonoid supplements. Therefore, it is also difficult to associate the protective effects on the immune system function from flavonoids with better performances.

Apart from these three studies that clearly demonstrate the improvement of immune functions with flavonoid intakes during exercise [52,67,98], other studies have demonstrated a certain anti-inflammatory activity from flavonoid consumption [59,65,82,85], and others failed to demonstrate the protective effects of flavonoids on inflammatory biomarkers [26,50,56,60,61,77,91,97]. It remains to be found what happened in the in vivo function of athletes or recreational subjects recruited in these studies, because the incidence of URTIs was not determined in such articles. Only Nieman et al. performed a wide study of biomarkers of the immune system, demonstrating that, with the administration of quercetin, the plasma inflammatory cytokines did not vary [59] or the NK cell activity, lymphocyte proliferative capacity, or salivary IgA [52], but there was a clear decrease in URTI incidence in these athletes [52]. Similarly, the same group demonstrated, in another clinical trial, there was no effect of the flavonol intake on the plasma inflammatory cytokines [97] but an increase in the antiviral activity, with no effect on the antibacterial potential [98]. Therefore, these studies allow the suggestion that plasma inflammatory biomarkers and even salivary IgA are not good predictors of what can eventually occur during the risk of infections after exercise bouts.

Comment must be made on the risk of bias in the studies included here. Although the overall assessment of bias of the included studies was low, it should not be forgotten that, in two domains, the number of articles with a low bias was around 25%. A more detailed description of the randomization process of some of the studies, as well as a more specific explanation of the allocation concealment, would allow a higher number of articles with a

selection bias rated as low. Moreover, we would like to highlight that, in the case of some sponsored studies, no detailed description of the potential influence of the sponsors over the results were included.

Finally, although this review summarizes the current knowledge about the effects of flavonoids on the exercise performance and exercise-induced changes in immune and inflammatory biomarkers, some limitations need to be considered. Whereas some of the reviewed studies focused exclusively on exercise performance assessment, others reported it as a secondary outcome. For this reason, the performance assessment was established by different variables. It would be interesting if articles provided the same performance measures for a better evaluation of the flavonoid ergogenic effect—for instance, in running and cycling, the time to cover a certain distance would be very objective and easy to compare between different studies. Moreover, the included interventions were very heterogeneous; depending on the study, the flavonoid supplementations were provided in everyday foods or in dietary supplements that could influence the flavonoid absorption. In addition, the intrinsic individual factors, such as human subjects' genetics and gut microbiota composition and functionality [145], are recognized to affect the flavonoid absorption and, eventually, influence its body effects. The doses also varied between the studies and flavonoid subclass, the average being about 430 mg/day, which could be achieved in a natural way by fruit intake. In addition, it should be noted that most of the studies were performed with recreationally active participants, trained people or even elite athletes, and the outcome may depend on the fitness level of the participants and the kind of exercise performed.

5. Conclusions

Although promising results have been found regarding flavonoid supplementation in sports performances, no clear conclusions can be drawn. The intake of anthocyanin-enriched extracts seems to be the most promising strategy for both enhancing physical performances and counteracting the increase in inflammation induced by intensive exercise, although further studies are encouraged to confirm these effects, establish the optimal dosage, elucidate the dose-response effect, and ascertain their impact on the immune status.

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OBJECTIVES

Over the recent years, the participation in intensive sport events has increased, raising concerns about the potential harmful impact of exhausting exercise on immune function and how to prevent it. Besides following an adequate training and specific dietary recommendations, some bioactive compounds have been proposed as potential nutraceuticals for improving exercise performance, preventing oxidative stress and enhancing immune function. Among them, polyphenols have been given increasing attention lately. Although they started to be studied for their intrinsic antioxidant properties, they have demonstrated many other beneficial effects, including their cardioprotective, anti-inflammatory and immunomodulatory properties. Cocoa is one of the best sources of polyphenols among commonly consumed food items. Furthermore, cocoa contains other bioactive compounds with immunomodulating properties, such as a high proportion of fibre and methylxanthines. Hesperidin, the main flavonoid in citrus fruits and one of the most consumed polyphenols, has also demonstrated many health benefits, among them, the modulation of the oxidative status after intensive exercise.

Based on this background, the hypothesis that supports the current thesis is that intensive exercise can impair immune function while a diet rich in polyphenols and/or dietary fibre may prevent these changes.

The main goals of the current thesis were i) **to establish the immune alterations induced by intensive exercise** and ii) **to evaluate the possible preventive properties of cocoa, cocoa fibre and hesperidin on such alterations, focusing on the changes induced in mucosal immunity as well as those induced in systemic immunity.**

To achieve these main goals, the following specific objectives were proposed:

1. **To evaluate the influence of both intensive and exhausting exercise on the gut microbiota composition and the mucosal immune system**.

   The results obtained from the first objective are part of the following publications:

   Article 1: Ruiz-Iglesias P.; Massot-Cladera, M.; Estruel-Amades, S.; Pérez-Cano, FJ.; Castell, M. "Intensive training and sex influence intestinal microbiota composition: a preclinical approach". *Proceedings* 2020, 61, 11.

Article 2: Ruiz-Iglesias, P.; Estruel-Amades, S.; Camps-Bossacoma, M.; Massot-Cladera, M.; Castell, M.; Pérez-Cano, F.J. "Alterations in the mucosal immune system by a chronic exhausting exercise in Wistar rats". *Scientific Reports* 2020, 10, 17950.

2. **To assess the influence of cocoa, hesperidin and their combination in the immune system alterations induced by intensive and exhausting exercise.**

The results obtained from the second objective are part of the following publications:

Article 3: Ruiz-Iglesias P.; Massot-Cladera, M.; Rodríguez-Lagunas, MJ.; Franch, A.; Camps-Bossacoma, M.; Pérez-Cano, FJ.; Castell, M. "Protective effect of a cocoa-enriched diet on oxidative stress induced by intensive acute exercise in rats". *Antioxidants* 2022, 11, 753.

Article 4: Ruiz-Iglesias P.; Massot-Cladera, M.; Rodríguez-Lagunas, MJ.; Franch, A.; Camps-Bossacoma, M.; Castell, M.; Pérez-Cano, FJ. "A cocoa diet can partially attenuate the alterations in microbiota and mucosal immunity induced by a single session of intensive exercise in rats". *Frontiers in Nutrition* 2022, 9, 861533.

Article 5: Ruiz-Iglesias P.; Estruel-Amades, S.; Camps-Bossacoma, M.; Massot-Cladera, M.; Franch, A.; Pérez-Cano, FJ.; Castell, M. "Influence of hesperidin on systemic immunity of rats following an intensive training and exhausting exercise". *Nutrients* 2020, 12, 1291.

Article 6: Ruiz-Iglesias P.; Massot-Cladera, M.; Pérez-Cano, FJ.; Castell, M. " Dietary interventions with cocoa and hesperidin on the systemic immunity of intensively trained and exhausted rats". *Submitted.*

RESULTS

ARTICLE 1

"Intensive training and sex influence intestinal microbiota composition: a preclinical approach"

Patricia Ruiz-Iglesias, Malén Massot-Cladera, Sheila Estruel-Amades, Francisco J. Pérez-Cano, Margarida Castell.

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ABSTRACT

Aim: To establish the effect of intensive exercise training followed by exhausting exercise on gut microbiota composition and, at the same time, to ascertain the influence of sexual dimorphism in such changes.

Methods: For this purpose, male and female Wistar rats were submitted to a running training programme that involved 2 weeks of running once a day with increasing speed, 2 weeks of running twice a day for 25-30 min at 25-30 m/min (6 h between training sessions) and a final exhaustion test. Parallel groups of female and male sedentary (SED) rats (age matched) were included. Caecal samples were collected after performing the final exhaustion test to characterize the microbiota composition by 16S rRNA sequencing.

Results: Neither the exercise training nor the sexual dimorphism induced changes on the microbial diversity and richness indexes. With regard to sex-induced changes in microbial relative abundance, male rats had a higher proportion of the phylum Actinobacteria, the family *Bifidobacteriaceae* and *Bifidobacterium* spp., while females had increased proportions of *Odoribacteraceae* (belonging to Bacteroidetes), *Clostridiaceae* and *Eubacteriaceae* (both Firmicutes) families. After exercise, the percentage of *Bifidobacterium* spp. was decreased in male rats. In female rats, exercise induced a reduction in the proportion of the family *Eubacteriaceae* and an increase in the proportion of the genus *Paraeggerthella*. Moreover, qualitative changes in caecal microbiota composition were observed after intensive training and exhausting exercise. Some families were just present in sedentary rats while others colonized the caecal microbiota after exercise, among them, the family *Staphylococcaceae* was exclusively present in male runner rats and the family *Coriobacteriaceae* was only found in female runner rats.

Conclusion: Intensive training and sexual dimorphism influence the caecal microbiota composition in Wistar rats at both quantitative and qualitative level.

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Intensive Training and Sex Influence Intestinal Microbiota Composition: A Preclinical Approach †

Patricia Ruiz-Iglesias [1,2], Malén Massot-Cladera [1,2], Sheila Estruel-Amades [1,2], Francisco J. Pérez-Cano [1,2,*] and Margarida Castell [1,2,*]

[1] Section of Physiology, Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science, University of Barcelona (UB), 08028 Barcelona, Spain; patriciaruiz@ub.edu (P.R.-I.); malen.massot@ub.edu (M.M.-C.); sheila199134@gmail.com (S.E.-A.)

[2] Nutrition and Food Safety Research Institute (INSA-UB), Torribera Campus, Faculty of Pharmacy and Food Science, UB, 08921 Santa Coloma de Gramenet, Spain

* Correspondence: franciscoperez@ub.edu (F.J.P.-C.); margaridacastell@ub.edu (M.C.)

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Abstract: Lifestyle, including regular physical activity and dietary habits, influences microbiota composition. Although some studies have demonstrated changes in microbiota composition due to moderate or high intensity training in athletes, the relationship between physical activity and changes in the intestinal bacteria is still a matter of discussion. In addition, as most studies are performed on males, the role of sexual dimorphism deserves to be explored. Therefore, the aim of this preliminary study was to ascertain the influence of an intensive training and the rat's sex on caecal microbiota composition. For this purpose, female and male 4-week-old Wistar rats were submitted to a 4-week running training program. At the end, caecal samples were collected immediately after performing an exhaustion test to characterize the microbiota composition by 16S rRNA sequencing technique. Parallel groups of female and male sedentary (SED) rats (age matched) were included. The results showed that young female rats had a higher ability to run than males but no sex- or training-associated changes were observed on microbiota diversity and richness indexes among groups. However, the Actinobacteria, *Bifidobacteriaceae* and *Bifidobacterium* spp. proportions were significantly higher in male than in female rats when comparing SED groups ($p < 0.05$), whose proportions in males were decreased by the training program ($p < 0.05$ vs. SED). On the contrary, female SED rats showed a higher proportion of *Odoribacteraceae* (belonging to Bacteroidetes), *Clostridiaceae* and *Eubacteriaceae* (both Firmicutes) than in the respective male group ($p < 0.05$), although *Eubacteriaceae* proportion decreased by running ($p < 0.05$ vs. SED). However, training increased the proportion of the *Paraeggerthella* genus (Actinobacteria) in female rats with respect to its sedentary counterpart ($p < 0.05$). Overall, caecal microbiota composition is modified by intensive training in young rats but there are also sex-based differences that should be considered for interventional studies.

Keywords: exercise; training; running; microbiota; dimorphism

1. Introduction

The modulatory role of the intestinal microbiome composition on physical activity has been described in some studies, which have shown an association with increased biodiversity and the presence of bacterial groups with beneficial functions [1]. However, other studies have not been able to find such a correlation and, for instance, a recent study in lean and overweight men showed the lack of effect of a short-term high-intensity interval training exercise on gut bacterial diversity or

composition [2]. For this reason, the relationship between physical activity and changes in the intestinal bacteria is still a matter of discussion [3]. In addition, the few studies performed aiming to establish this link between exercise and microbiota are mainly performed in male athletes. Although diet is one of the strongest factors influencing gut microbiota composition [4], sex differences can also have an impact on that. Thus, as microbiota composition is suggested to be different in males and females [5], the differential response to exercise should be explored in both sexes.

In order to deep into the effect on microbiota composition of these two variables, exercise and sex, we used a preclinical approach based on an intensive training design in rats which has been demonstrated in previous studies to have an impact on rat physiology [6,7]. Particularly, the acute exercise performance with increasing duration and speed induced some changes in body weight, intestinal features and the immune status of both innate and acquired immune response [6,7]. The aim of this study was to ascertain the influence of an intensive training and the rat's sex on the caecal microbiota composition.

2. Material and Methods

2.1. Animals and Training Program

For this purpose, 4-week-old male and female Wistar rats (n = 22) from Envigo (Blackthorn, UK) were housed in the animal facilities of the Faculty of Biology of the University of Barcelona (UB) in polycarbonate cages (2–3 rats per cage) in a controlled environment of temperature and humidity, in a 12/12 h light/dark cycle with free access to water and food. The sample size used in the study was established by the Appraising Project Office's program from the Universidad Miguel Hernández (Elche, Spain).

The training program was achieved by means of a LE8700 treadmill (Panlab, Harvard, MA, USA) and an Exer3/6 treadmill (Columbus, OH, USA) with same length and width for each running lane, following an exercise program design as previously described [6,7]. Firstly, two periods of habituation were included: a 3-day period on a turned-off treadmill and a 5-day period in treadmills with increasing duration and speed (RUN group) (Figure 1). After selection, the RUN group began a period in which the duration and the speed was increased progressively (10 min/session at 5 m/min to a 25 min/session at 25 m/min). In the following 2 weeks, intensive training started and RUN animals ran twice a day (30 min at 30 m/min, 6 h between sessions), 5 days per week. During the experiment, age and sex matched sedentary animals (SED group) were also placed in the static treadmill receptacle. Thus, the four experimental groups were as follows: female RUN (n = 6), female SED (n = 5), male RUN (n = 6), male SED (n = 5). After the daily exercise, rats from both RUN and SED groups received a 50% solution of condensed milk (100 μL/100 g body weight) as a reward.

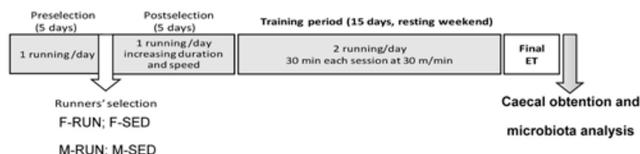

Figure 1. Experimental design of the intensive training program. Groups: female runners (F-RUN, n = 6), female sedentary (F-SED, n = 5), male runners (M-RUN, n = 6), male sedentary (M-SED, n = 5). At the end of the training RUN animals were subjected to a final exhaustion test (ET).

At the end of the 2 weeks, an exhaustion test (ET) was performed, starting with an initial speed of 5 m/min with a progressive increase of 1.8 m/min every min until exhaustion. Immediately after

the final exhaustion test, animals were euthanized with ketamine (Merial Laboratories S.A., Barcelona, Spain) / xylazine (Bayer A.G., Leverkusen, Germany) and caecal samples were collected.

The experimental procedure was validated by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEEA/UB ref. 464/16 and DAAM 9257, respectively).

2.2. Microbiota Determination

Three samples from each group were selected to characterize the microbiota composition by 16S rRNA sequencing as in previous studies [8]. Briefly, genomic DNA was extracted from caecal samples (n = 3/group) using the DNeasy Blood and Tissue Mini kit (Qiagen, Madrid, Spain) and amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina Inc, San Diego, CA, USA). Image analysis, base calling and data quality assessment were performed in the MiSeq instrument. Sequences were merged and processed using Pair-End read merger (PEAR v 0.9.6, Exelixis Lab, Heidelberg, Germany) and Cutadapt v1.8.1, as previously described [9]. The presence or absence of genera at a qualitative level was represented in a Venn diagram. A bacterial group was considered as present by establishing a cutoff of 3 animals displaying proportions higher than 0.001%, while the bacterial groups not detected in any of the animals were regarded as absent.

2.3. Statistical Analysis

For statistical analysis, the Statistical Package for the Social Sciences (SPSS v22.0) (IBM, Chicago, IL, USA) was used. Homogeneity of variance and normality distribution were tested by the Levene's and Shapiro–Wilk tests, respectively. When data were homogeneous and had a normal behavior, Student t-test was used to analyze statistical differences. Otherwise, the Mann–Whitney U test was performed. Significant differences were established when $p < 0.05$.

3. Results and Discussion

The results showed no sex- or training-associated changes on microbiota diversity and richness indexes among groups. However, some changes in relative abundances were observed. At phylum level, only the Actinobacteria proportion was associated with sex or exercise condition. This change was in line with changes in the family *Bifidobacteriaceae* and the genus *Bifidobacterium* spp. All these proportions were significantly higher in male rats than in female rats when comparing SED groups ($p < 0.05$), and the proportion in males decreased by the training program ($p < 0.05$ vs. SED) (Figure 2).

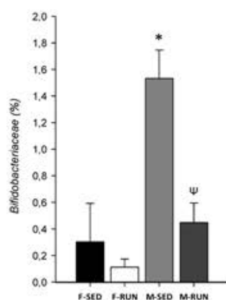

Figure 2. Relative abundance of the family *Bifidobacteriaceae* in caecal content at the end of the exhaustion test differentiating between sexes and exercise condition. Groups: female runners (F-RUN), female sedentary (F-SED), male runners (M-RUN), male sedentary (M-SED). Results are expressed as mean ± SEM (n = 3/group). Statistical significance: * $p < 0.05$ male vs. female with same exercise condition, $^{\Psi} p < 0.05$ RUN vs. SED with same sex condition.

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On the contrary, female SED rats showed a higher proportion of *Odoribacteraceae* (belonging to Bacteroidetes), *Clostridiaceae* and *Eubacteriaceae* (both Firmicutes) than in the respective male group ($p < 0.05$), although the *Eubacteriaceae* proportion decreased through running ($p < 0.05$ vs. SED). However, training increased the proportion of *Paraeggerthella* genus (Actinobacteria) in female rats with respect to its sedentary counterpart ($p < 0.05$).

The analysis of the genera distribution in Venn diagrams at family level revealed that there was a core of 30–32 genera that persisted between all four experimental groups when considering both sexes and exercise conditions separately (Figure 3).

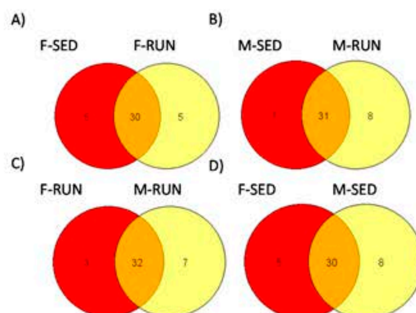

Figure 3. Venn diagrams showing the diversity in all families differentiating between exercise condition (**A, B**) and sexes (**C, D**). Results derived from n = 3/group. Groups: female runners (F-RUN), female sedentary (F-SED), male runners (M-RUN), male sedentary (M-SED).

Moreover, when comparing the microbiota depending on the sex or the exercise, it could be observed that specific families were particularly associated with a certain condition (Figure 3). In this sense, the training and exhaustion test led to the colonization of new genera, such as *Chromobacteriaceae, Coriobacteriaceae, Methylococcaceae, Phyllobacteriaceae* and *Rhodobacteraceae* in the case of females and *Erwiniaceae, Intrasporangiaceae, Phyllobacteriaceae, Rhizobiaceae, Rhodanobacteraceae, Rhodobacteraceae, Spongiibacteraceae* and *Staphylococcaceae* in the case of males. Regarding the sex differential presence of bacterial groups, *Aerococcaceae, Atopobiaceae, Chromobacteriaceae, Comamonadaceae, Coriobacteriaceae, Pasteurellaceae, Planococcaceae* and *Xanthomonadaceae* were exclusively present in males but not in females at sedentary conditions, whereas *Atopobiaceae, Erwiniaceae, Intrasporangiaceae, Pasteurellaceae, Planococcaceae, Rhodanobacteraceae* and *Spongiibacteraceae* appeared in males but not in females after the training and exhaustion test.

4. Conclusions

Overall, the present study evidenced that the caecal microbiota composition is modified by intensive training in young rats at a relative abundance and qualitative level. In addition, some sex-based differential responses against the exercise were found. Thus, sex is a variable that should be considered for interventional studies in the future concerning physical activity.

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Conflicts of Interest: The authors declare no conflict of interest.

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ARTICLE 2

"Alterations in the mucosal immune system by a chronic exhausting exercise in Wistar rats"

Patricia Ruiz-Iglesias, Sheila Estruel-Amades, Mariona Camps-Bossacoma, Malén Massot-Cladera, Margarida Castell, Francisco J. Pérez-Cano.

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- **IV Workshop l'Institut de Recerca en Nutrició i Seguretat Alimentària de la Universitat de Barcelona (INSA-UB)**. Barcelona, November 2018. Ruiz-Iglesias P.; Estruel-Amades, S.; Camps-Bossacoma, M.; Périz, M.; Franch, À.; Pérez-Cano, FJ.; Castell, M.; Massot-Cladera, M. "**Alterations in the intestinal immune system by a chronic exhausting exercise in rats**".

ABSTRACT

Aim: To characterize the alterations on mucosal immune system induced by a rat model of chronic intensive training alone or followed by exhausting exercise.

Methods: Female Wistar rats were randomized into two groups: runner and sedentary. The runner group undergone an intensive running training programme including three trainings per week and two exhaustion tests. After 5 weeks, samples from the runner group were obtained either before (T), immediately after (TE) or 24 h after (TE24) performing an additional exhaustion test to assess the influence of exercise on the immune status at different timepoints.

Results: The intensive training reduced the proportion of B cells in mesenteric lymph nodes and decreased the concentration of salivary immunoglobulin A. In addition, the intensive training downregulated the small intestine gene expression of claudin-4 and occludin, whereas induced a more than three-fold increase in claudin-2 expression. The final exhaustion impaired the gene expression of zonula occludens proteins and enhanced the interleukin-2 and interferon-γ secretion by mesenteric lymph nodes lymphocytes.

Conclusion: Intensive training for 5 weeks followed or not by an additional exhaustion alters the mucosal immune system and the intestinal epithelial barrier function in Wistar rats.

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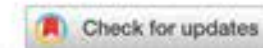

OPEN

Alterations in the mucosal immune system by a chronic exhausting exercise in Wistar rats

Patricia Ruiz-Iglesias[1,2], Sheila Estruel-Amades[1,2], Mariona Camps-Bossacoma[1,2], Malén Massot-Cladera[1,2], Margarida Castell[1,2,3]✉ & Francisco J. Pérez-Cano[1,2]✉

Exhausting exercise can disturb immune and gastrointestinal functions. Nevertheless, the impact of it on mucosal-associated lymphoid tissue has not been studied in depth. Here, we aim to establish the effects of an intensive training and exhausting exercise on the mucosal immunity of rats and to approach the mechanisms involved. Rats were submitted to a high-intensity training consisting of running in a treadmill 5 days per week for 5 weeks, involving 2 weekly exhaustion tests. At the end, samples were obtained before (T), immediately after (TE) and 24 h after (TE24) an additional final exhaustion test. The training programme reduced the salivary production of immunoglobulin A, impaired the tight junction proteins' gene expression and modified the mesenteric lymph node lymphocyte composition and function, increasing the ratio between Tαβ+ and B lymphocytes, reducing their proliferation capacity and enhancing their interferon-γ secretion. As a consequence of the final exhaustion test, the caecal IgA content increased, while it impaired the gut zonula occludens expression and enhanced the interleukin-2 and interferon-γ secretion. Our results indicate that intensive training for 5 weeks followed or not by an additional exhaustion disrupts the mucosal-associated lymphoid tissue and the intestinal epithelial barrier integrity in rats.

The mucosal immune system is the largest immune component of the body, shaped by the mucosa-associated lymphoid tissue (MALT) where about half of the whole lymphocyte population is found[1]. MALT cells are scattered along the surfaces of all mucosal tissues and constitute the starting point for a great number of immune responses because of its constant exposure to antigens. In addition, immune responses developed in a particular MALT structure will influence the immunity of the entire MALT due to its property of recirculating immune cells between mucosa and glands[2]. MALT comprises, among others, the salivary duct-associated lymphoid tissue (DALT) and the gut-associated lymphoid tissue (GALT), which defends the gastrointestinal tract against infections[1]. One of the main effector functions of the MALT is to produce and secrete immunoglobulin A (IgA)[1].

It is well known that regular bouts of moderate-intensity exercise offers several long-term health benefits[3], such as preventing, delaying or improving the prognosis of several chronic diseases[4], and even cancer[5], enhancing immunity[6] and inducing benefits for the gastrointestinal (GI) tract[7] and the gut microbiota[8]. However, chronic intensive exercise can induce adverse effects on health, such as oxidative stress, muscle damage and inflammation[9], as well as immune[10] and GI[11] alterations. The increasing participation of the general population in endurance events over the last decades has raised concerns regarding the impact of prolonged overly intense exercise on immune and GI health[11,12]. Focusing on the immune system, the impact of physical activity also depends on the intensity and duration of the effort. Regular bouts of moderate exercise enhance immune function, whereas strenuous exercise may impair it, decreasing host protection accordingly and leading to a higher risk of GI and upper-respiratory tract infections (URTIs) 1–2 weeks after a competition[13] and to a lower performance[14]. The appearance of GI symptoms related to excessive exercise has been reported to be 30–93% among distance runners and triathletes[15–17]. Most of them are mild and do not cause long-term health effects (epigastric pain, heartburn, nausea, vomiting, abdominal pain and diarrhoea), but oesophagitis, haemorrhagic gastritis, gastric ulcer, gastrointestinal bleeding and ischaemic bowel may involve severe medical complications[16,18,19]. The underlying mechanisms are not fully understood but this symptomatology seems to be mainly related to GI ischaemia, altered motility, malabsorption and neuroendocrine factors[11,20].

[1]Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), Av. Joan XXIII 27-3, 08028 Barcelona, Spain. [2]Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), UB, 08921 Santa Coloma de Gramenet, Spain. [3]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain. ✉email: margaridacastell@ub.edu; franciscoperez@ub.edu

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Week	Maximum running time (min)		
	Monday ET	Friday ET	p value
1	23.83 ± 0.51	24.70 ± 0.55	NS
2	23.83 ± 0.61	25.17 ± 0.81	NS
3	22.26 ± 0.70*	24.22 ± 0.92	0.042
4	21.18 ± 0.94*	23.48 ± 0.59	0.003
5	21.48 ± 0.76*	22.30 ± 0.67#	NS

Table 1. Maximum running time lasted in the Monday and Friday exhaustion tests performed throughout the high-intensity exercise training programme. *ET* exhaustion test, *NS* no statistically significant differences detected. Data are expressed as mean ± SEM (n = 8). Statistical differences (paired Student *t* test): significant differences between consecutive Monday ET and Friday ET are included in the table ($p < 0.05$); *significant differences vs the first two Monday ETs ($p < 0.05$); #significant differences vs the first three Friday ETs ($p < 0.05$).

The measurement of salivary IgA concentration in humans is one of the most used biomarkers to assess the effect of exercise on mucosal humoral immunity[21]. In particular, regular sessions of moderate exercise enhances salivary IgA secretion[22], whereas prolonged periods of intensive exercise may decrease it, contributing, at least in part, to the higher susceptibility to infections observed in athletes[21]. On the other hand, exercise also impacts cellular immunity[21]. Most of the studies have assessed changes in blood lymphocytes, whereas only a few have focused on lymphoid compartments such as bone marrow, Peyer's patches[21], spleen, thymus[23] and lymph nodes[24]. The changes in the proportion of lymphocyte populations that have been reported in such tissues may reflect a redistribution of cells among lymphoid tissues which must be mainly due to the release of stress hormones such as catecholamines and glucocorticoids[25]. In addition, changes in the functionality of natural killer (NK), T and B lymphocytes have been described[23,26], as well as in the T helper (Th)1/Th2 cell balance[27] in both blood and lymphoid tissues. Previous studies have evidenced the effect of exercise on axillary, inguinal and submandibular lymph nodes' specific immunity[24], but, to our knowledge, the impact of high-intensity exercise in the GALT, and particularly in mesenteric lymph nodes (MLNs), remains uncertain. MLNs belong to the organized GALT and play an important role in the development of local immune responses in the gut[28]. Therefore, alterations in MLN lymphocyte (MLNL) composition and function may contribute to the explanation of the mechanisms underlying exercise-induced gastrointestinal syndrome.

Despite the existing research, the isolated role of exercise in disrupting GI and immune function has been challenged lately, since increasing evidence suggests that many other uncontrolled factors, such as anxiety, sleep deprivation, travel, nutritional deficits, environmental extremes and exposure to pathogens by attending a mass participation event may be involved[6]. Therefore, a well-controlled animal model of intensive exercise may be useful to elucidate the impact of exercise per se on the immune system, and, more specifically, on the MALT. Previously, we have characterized the alterations occurring in the innate and adaptative immune system as well as in the oxidative status in high-intensity trained rats[23,26,29]. The present study aimed to establish the impact of intensive training and exhaustion exercise on the mucosal immune system functionality of rats and to approach the mechanisms involved.

Results

Performance. The high-intensity training programme involved 5 days of training per week for 5 weeks: 3 regular trainings on Tuesday, Wednesday and Thursday, and an exhaustion test (ET) every Monday (M) and Friday (F), in which maximum running time indicated rat physical performance (Table 1). Fridays' performance was better than that on Mondays ($p < 0.05$ for weeks 3 and 4). Rats supported a maximum time of about 25 min on the second Friday, achieving a maximum speed of about 59.0–71.8 m × min^{-1}. Later, in weeks 3–5, the performance achieved on Monday was lower than that on Mondays from previous weeks ($p < 0.05$), which was also observed on the last Friday in comparison with the previous ones ($p < 0.05$). An additional ET was performed after the 5-week high-intensity training programme, where trained rats ran for 32.07 ± 1.50 min (mean ± standard error).

Body weight and food efficiency. Body weight and chow intake were monitored throughout the 5-week training programme (Fig. 1a,b) in both runners and sedentary (SED) rats. Although the body weight was similar during the first weeks of training, runner animals showed a higher body weight than SED animals on the last days of the study ($p < 0.05$). This increase was not associated with a higher chow intake, quite the opposite: runner animals showed a lower weekly chow intake than SED rats from the beginning of the training programme ($p < 0.05$ in weeks 1–4).

Body weight gain and chow intake allowed the calculation of the week-long food efficiency (Fig. 1c). The pattern of food efficiency progression during the study was similar between SED and runner animals, substantially increasing during the first 2 weeks, probably due to the young age of the animals. The high-intensity training tended to increase the food efficiency throughout the study, the difference being statistically significant only in the second week of the training programme ($p = 0.008$).

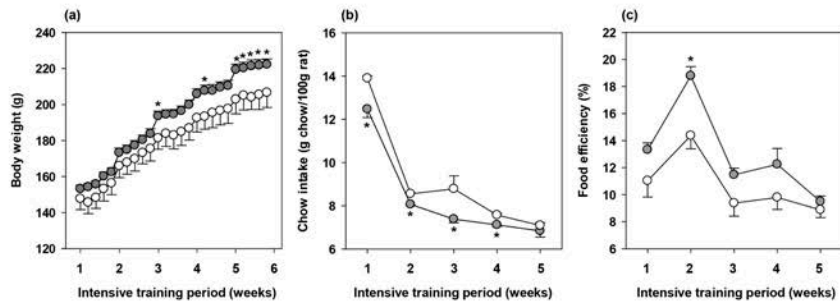

Figure 1. Body weight (**a**), chow intake (**b**), and food efficiency (**c**) throughout the intensive training period. The sedentary (SED) group is represented by white symbols (○) and the runner group by grey symbols (◎). Data are expressed as mean ± standard error (n = 8 animals for the SED group and n = 24 animals for the runner group in (**a**); n = 3 cages for the SED group and n = 8 cages for the runner group in **b**,**c**). Statistical differences (Student's t test): *p < 0.05 vs SED group.

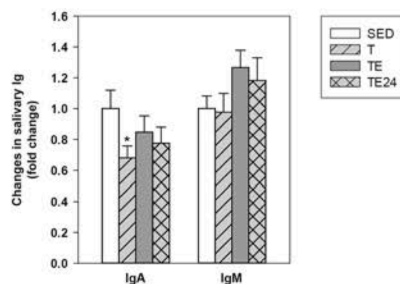

Figure 2. Changes in submaxillary salivary gland IgA and IgM concentration compared to the sedentary group. *SED* sedentary rats, *T* trained rats, *TE* T rats immediately after a final exhaustion test, *TE24* TE rats 24 h after the final exhaustion test. Data are expressed as mean ± standard error (n = 8). Statistical differences (one-way ANOVA followed by post-hoc Tukey test): *p < 0.05 vs SED group.

Salivary gland immunoglobulins. After the 5-week intensive training, in order to assess the mucosal immune status at different time points, runner rats were divided into 3 groups according the conditions of sample collection: T group, whose sample were collected one day after performing a regular training, TE group, whose samples were obtained immediately after an additional final exhaustion test, and TE24 group whose samples were collected 24 h after the additional final exhaustion test. The results from these rats were compared with those obtained from matched sedentary rats (SED).

Content of IgA and IgM was determined in submaxillary salivary glands (SMGs) (Fig. 2). The intensive training induced a decrease in the IgA content in this compartment (p = 0.05, T group vs SED group) that was not statistically significant after carrying out the additional final ET. Neither training nor exhaustion induced significant changes in salivary IgM concentration, although exhaustion tended to increase it in comparison to sedentary animals (p = 0.098, TE group vs SED group).

Intestinal immunoglobulins. Intestinal IgA concentration was determined in gut washes (GWs) from the small intestine and in caecal content (CC) (Fig. 3a,b). In the GWs, neither training nor exhaustion induced changes in IgA content, although values tended to decrease 24 h after the final exhaustion test without reaching significance. With regard to the CC, IgA levels tended to decrease after 5 weeks of intensive training (T group) but, after the final exhaustion test, there was a higher IgA content that remained for at least 24 h (p = 0.018, T group vs TE group; p = 0.009, T group vs TE24 group).

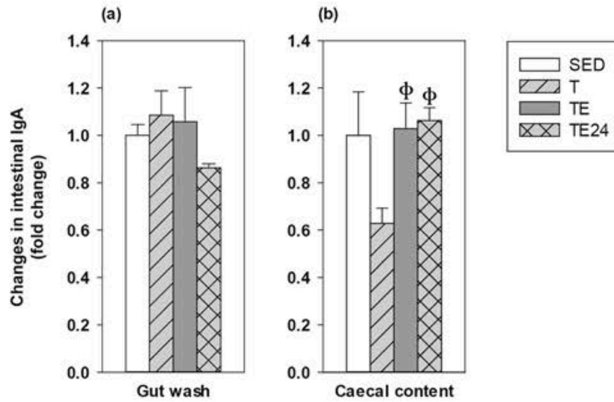

Figure 3. Changes in gut wash IgA (**a**), and caecal content IgA (**b**) concentration compared to the sedentary group. *SED* sedentary rats, *T* trained rats, *TE* T rats immediately after a final exhaustion test, *TE24* TE rats 24 h after the final exhaustion test. Data are expressed as mean ± standard error (n = 8). Statistical differences (Kruskal–Wallis followed by Mann–Whitney *U* test): $^{\phi}p < 0.05$ vs. T group.

Gene	SED	T	TE	TE24
Oclud	1 ± 0.09	0.81 ± 0.04*	0.86 ± 0.08	1.09 ± 0.01$^{\phi}$
Cldn-2	1 ± 0.17	2.97 ± 0.43*	3.98 ± 0.70*	2.70 ± 0.54*
Cldn-4	1 ± 0.13	0.62 ± 0.05*	0.97 ± 0.07$^{\phi}$	1.19 ± 0.23$^{\phi}$
ZO-1	1 ± 0.11	1.12 ± 0.10	1.76 ± 0.23$^{*\phi}$	1.25 ± 0.16$^{\lambda}$
ZO-2	1 ± 0.05	0.88 ± 0.03	0.75 ± 0.07$^{*\phi}$	1.10 ± 0.05$^{\phi\lambda}$
pIgR	1 ± 0.15	1.08 ± 0.08	0.83 ± 0.13	0.87 ± 0.14
TGF-β	1 ± 0.20	0.64 ± 0.09	0.75 ± 0.13	0.60 ± 0.04
RAR-α	1 ± 0.07	0.83 ± 0.05	0.75 ± 0.04*	0.89 ± 0.06
CCL25	1 ± 0.08	1.02 ± 0.06	0.97 ± 0.11	0.96 ± 0.10

Table 2. Changes in gene expression of some molecules in small intestine compared to the sedentary group. *SED* sedentary rats, *T* trained rats, *TE* T rats with a final exhaustion test, *TE24* TE rats 24 h after the final exhaustion test. Data are expressed as mean ± standard error (n = 8). Statistical differences (Kruskal–Wallis followed by Mann–Whitney *U* test): $^{*}p < 0.05$ vs SED group; $^{\phi}p < 0.05$ vs T group; $^{\lambda}p < 0.05$ vs TE group.

Small intestine gene expression and gut permeability. Alterations due to the intensive training programme and the final exhaustion in the gene expression of proteins involved in the gut homeostasis, including tight junction proteins (such as occludin, claudin-2, -4, zonula occludens-1, -2) and proteins involved in the B cell differentiation, IgA transcytosis and lymphocyte gut homing, were established (Table 2).

In the case of the tight junction proteins, the 5-week training programme decreased occludin (Oclud) and claudin (Cldn)-4 expression (p = 0.044 and p = 0.007, respectively, T group vs SED group), although both levels increased after performing the additional final ET. The claudin-4 expression found in the TE24 group inversely correlated with the performance achieved in the additional final ET (R = − 0.928, p = 0.008). In contrast, claudin-2 expression increased due to both intensive training and final exhaustion (p < 0.001 and p < 0.001, T group and TE group vs SED group), and these higher levels were maintained for at least 24 h (p = 0.011, TE24 group vs SED group).

In samples obtained immediately after the final exhaustion test (TE group) the expression of zonula occludens (ZO)-1 increased (p = 0.023, TE group *vs.* SED group) and that of ZO-2 decreased (p = 0.004, TE group *vs.* SED group) with respect to the sedentary rats. Both changes were normalized 24 h later.

With regard to the gene expression of proteins involved in B cell differentiation, IgA transcytosis and gut homing, no changes were found for polymeric immunoglobulin receptor (pIgR), transforming growth factor (TGF)-β1 and chemokine (C–C motif) ligand 25 (CCL25) due to the intensive training and/or exhaustion in

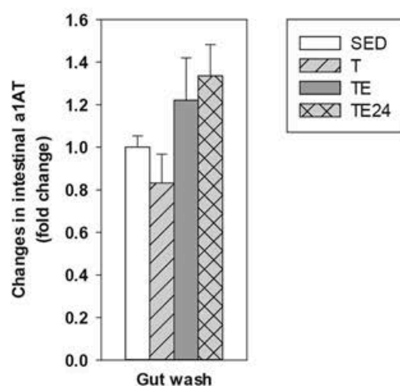

Figure 4. Changes in gut wash alpha-1-antytripsin (a1AT) concentration compared to the sedentary group. *SED* sedentary rats, *T* trained rats, *TE* T rats immediately after a final exhaustion test, *TE24* TE rats 24 h after the final exhaustion test. Data are expressed as mean ± standard error (n = 8).

comparison to sedentary animals. Nevertheless, the additional final ET decreased the retinoic acid receptor (RAR)-α gene expression. When considering the association of this results with the performance, there was an inverse correlation between the performance achieved in the additional final exhaustion test and the intestinal gene expression of TGF-β in the TE24 group ($R = -0.775$; $p = 0.041$).

On the other hand, alpha-1-antitrypsin (a1AT) concentration in GW was determined in order to assess changes in the gut paracellular permeability (Fig. 4). Although no significant changes in this marker were observed due to the 5 weeks of intensive training, the a1AT content in the GW tended to increase 24 h after carrying out the additional final ET ($p = 0.092$, TE24 group vs T group).

MLN lymphocyte composition. The influence of intensive training and the final exhaustion on the MLNL composition was also assessed (Fig. 5). In SED animals, MLNL included 33.10 ± 1.53% of B cells (CD45RA+), 57.45 ± 1.31% of Tαβ cells (TCRαβ+), 1.99 ± 0.19 of Tγδ cells (TCRγδ+) and 0.44 ± 0.04% of NK cells (CD161b+). Among all Tαβ cells, 75.04 ± 1.17% were Th (CD4+CD161b− in TCRαβ+), 25.13 ± 1.03% Tc (CD8+CD161b− in TCRαβ+) and 1.09 ± 0.05% NKT (CD161b+ in TCRαβ+) cells. The proportion of regulatory T (Treg) cells within CD4+ lymphocytes (CD25+FoxP3+ in CD4+) was 1.79 ± 0.11%.

The 5-week intensive training programme decreased the proportion of MLN B cells ($p = 0.008$, T group vs SED group) whereas it increased that of Tαβ lymphocytes ($p = 0.044$, T group vs SED group) (Fig. 5a). Consequently, the T/B cell ratio increased in the T group with respect to the SED group ($p = 0.011$) up to about 40% (Fig. 5b). After the final exhaustion test, the B and Tαβ cell proportions did not significantly differ from those of SED animals. No exercise condition modified the Tγδ and NKT cell proportions; however, in the TE group there was an inverse correlation between the time supported in the additional final exhaustion test and the proportion of MLN Tγδ cells ($R = -0.757$; $p = 0.049$).

With regard to the main Tαβ subsets, the MLN Tc cell proportion decreased in the T group ($p = 0.008$) compared to SED animals, which increased the Th/Tc ratio up to 20% (Fig. 5c,d). However, the proportion of Tc cells increased immediately after carrying out the additional final ET ($p = 0.004$, TE group vs T group), when there was a significant reduction of Th cell proportion ($p = 0.003$, TE group vs T group) and, as a result, a lower Th/Tc ratio than that observed in just trained rats ($p = 0.004$, TE group vs T group). All these changes concerning Tc and Th proportions in TE group were restored 24 h later, in the TE24 group.

Although none of the exercise conditions studied significantly modified the percentage of MLN Treg cells (Fig. 5e), there was an inverse correlation between the Treg proportion and the performance in the final exhaustion test ($R = -0.986$, $p < 0.001$ in the TE group and $R = -0.874$, $p = 0.005$ in the TE24 group).

MLN lymphocyte functionality. The proliferative response of MLN T-cells was established after concanavalin A (ConA) stimulation (Fig. 6a). After 5 weeks of intensive training, a lower proliferation capacity than that in the SED group was observed ($p = 0.045$, T group vs SED group). Nevertheless, it was overcome after carrying out the additional final ET ($p = 0.006$, TE group vs T group; $p = 0.003$, TE24 group vs T group).

Cytokine secretion was also quantified in MLNL culture supernatants (Fig. 6b–f). In SED animals, MLNL secreted 356.18 ± 50.94 pg/mL of interleukin (IL)-2, 1624.30 ± 469.29 pg/mL of interferon (IFN)-γ, 73.67 ± 12.92 pg/mL of IL-10, and 1.33 ± 0.19 pg/mL of IL-4 (mean ± standard error). The intensive training

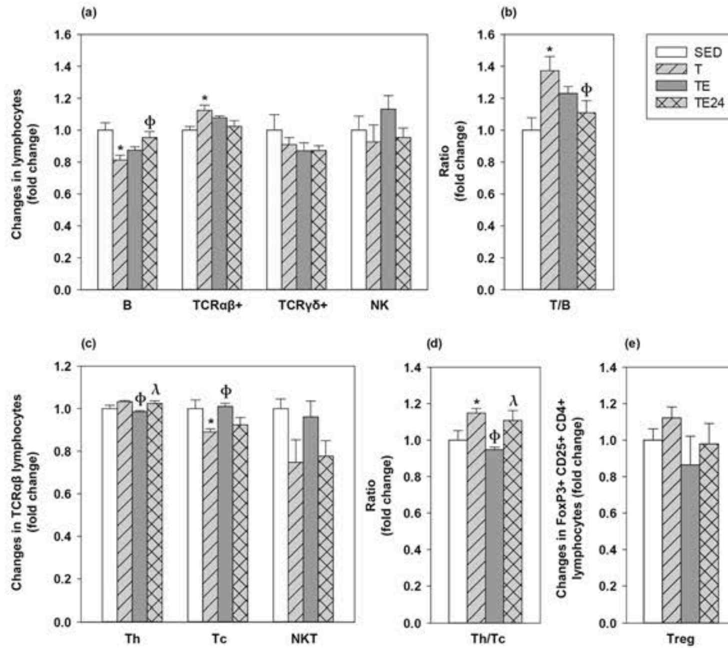

Figure 5. Changes in the proportion of mesenteric lymph node lymphocytes compared to the sedentary group. Main lymphocyte subsets (**a**); Tαβ/B lymphocytes ratio (**b**); main Tαβ subsets (**c**); Th/Tc ratio (**d**) and Treg proportion (**e**). *SED* sedentary rats, *T* trained rats, *TE* T rats with an additional final exhaustion test, *TE24* TE rats 24 h after the additional final exhaustion test. Data are expressed as mean ± SEM (n = 8). Statistical differences (one-way ANOVA followed by post-hoc Tukey test for (**a**,**b**) and Kruskal–Wallis followed by Mann–Whitney *U* test for **c**–**e**): *p < 0.05 vs SED group; $^{\phi}$p < 0.05 vs T group; $^{\lambda}$p < 0.05 vs TE group.

programme doubled the secretion of IFN-γ in MLNL (p = 0.005, T group vs SED group), and the final exhaustion test enhanced it even further (p < 0.001, TE group vs SED group), remaining elevated for at least 24 h (p < 0.001, TE24 group vs SED group). The final exhaustion test also increased the secretion of IL-2 in the TE (p = 0.003 vs SED group, p = 0.006 vs T group) and TE24 groups (p = 0.001 vs SED group, p = 0.002 vs T group). There were no significant changes regarding the secretion of IL-4 and IL-10 by MLNL, nevertheless, the levels of IL-10 positively correlated with the performance in the TE group (R = 0.921, p = 0.026).

The IFN-γ/IL-4 ratio was calculated in order to assess the Th1/Th2 balance (Fig. 6f). Both the TE and TE24 groups showed a significantly higher IFN-γ/IL-4 ratio (p = 0.001 and p = 0.006, respectively, vs SED group).

Finally, the in vitro ability for antibody production was determined in supernatants from non-stimulated MLNL (Fig. 7). In SED animals, MLNL in the assayed conditions produced 74.87 ± 10.39 ng/mL of IgA, 20.75 ± 5.54 ng/mL of IgM, and 17.81 ± 2.63 ng/mL of IgG (mean ± standard error). No changes were found in IgA and IgM secretion due to the training or the exhaustion test; however, IgG production remarkably increased in both the T and the TE groups (p = 0.014 and p = 0.009, respectively, vs SED group).

Discussion

Nowadays, it is widely accepted that strenuous exercise impairs the immune system and promotes gastrointestinal symptoms[10,11]. A growing amount of research is investigating the underlying mechanisms of exercise-induced gastrointestinal syndrome[11,30,31] and conclude that it is likely that this has a multifactorial cause, involving circulatory, enteric and immune alterations, as well as environmental triggers[11]. Efforts to link this symptomatology with intestinal barrier disruption due to splanchnic ischaemia have been relatively successful; nevertheless, the available literature on the mucosal immune system's contribution to these symptoms remains quite controversial. In previous studies, we demonstrated that rats submitted to intensive training and exhausting exercise exhibited

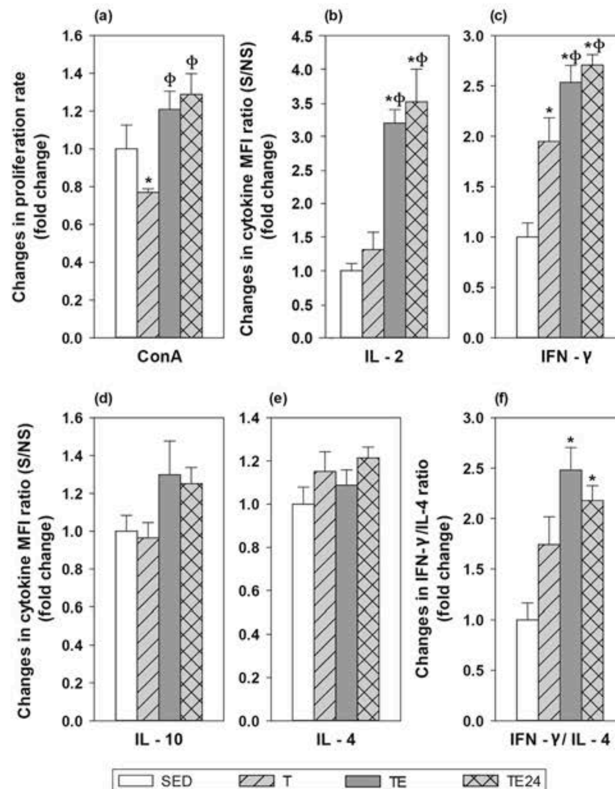

Figure 6. Changes in the proliferative response (**a**) and cytokine concentration released (**b–f**) by mesenteric lymph node lymphocyte stimulated by concanavalin A (ConA) compared to the sedentary group. *SED* sedentary rats, *T* trained rats, *TE* T rats with a final exhaustion test, *TE24* TE rats 24 h after the final exhaustion test. Data are expressed as mean ± standard error (n = 8). Statistical differences (one-way ANOVA followed by post-hoc Tukey test for **b**,**d**, and Kruskal–Wallis followed by Mann–Whitney *U* test for **a**,**c**,**e**): *p < 0.05 vs SED group; ᶲp < 0.05 vs T group.

alterations in both the innate and the acquired immunity[23,26]. The current study aimed to establish the effects of 5 weeks of intensive training on the mucosal immune system in rats and to elucidate the mechanisms underlying these effects.

Rats intensively trained for 5 weeks showed a better performance in the first 2 weeks but, in the last 2 weeks there was a clear lower performance, thus suggesting that animals may have achieved overtraining syndrome, which appears when the training workload exceed the body's ability to recover and this results, among other consequences, in a decrease in expected levels of performance[10]. Those runner animals showed a higher body weight gain, a lower chow intake and better food efficiency than sedentary animals throughout the training programme. This higher body weight (and consequently better food efficiency) could be due to an increase in muscle mass[32] and/or bone mass[33] and a decrease in fat content, as it has already been reported in female trained rats[34] and mice[35]. However no increases in body weight have been reported in male trained rodents[34–37]. These

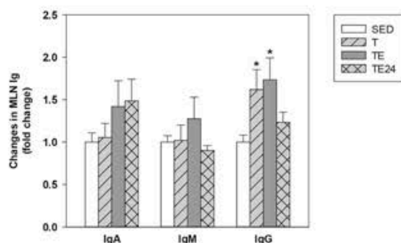

Figure 7. Changes in the IgA (**a**), IgM (**b**) and IgG (**c**) concentration in mesenteric lymph node lymphocyte supernatants compared to the sedentary group. *SED* sedentary rats, *T* trained rats, *TE* T rats with a final exhaustion test, *TE24* TE rats 24 h after the final exhaustion test. Data are expressed as mean ± standard error (n = 8). Statistical differences (Kruskal–Wallis followed by Mann–Whitney *U* test): *p < 0.05 vs SED group.

differences associated with rat gender deserve new studies aiming to establish the relationship between food intake, body weight and fat and muscle tissues in both male and female intensively trained rats.

Tight junction (TJ) proteins play a key role in maintaining the intestinal barrier function by regulating paracellular transport and secretion/absorption mechanisms. Claudins and zonula occludens proteins are vital for the TJ assembly and resistance, while occludin has a less vital regulatory role in the TJ[30]. There is evidence that the intestinal ischaemia-induced by exercise leads to an impairment of the gut epithelial barrier integrity, by means of TJ protein phosphorylation, disrupting the gut paracellular permeability[30,31]. Here, we found a decrease in occludin and claudin-4 gene expression due to the intensive training programme that could result in overtraining. Whereas these results agree with previous studies that found reduced occludin levels in intestinal cells in an in vitro heat stress model aiming to simulate exercise stress and recovery[38], they disagree with others that found similar expression levels in endurance exercise-trained rats and their sedentary counterparts[39], or even an increased occludin expression in obese rats submitted to high-intensity interval training[40]. To the best of our knowledge, changes in claudin-4 expression due to exercise have not been previously reported and they could have a relevant impact on GALT's functionality, since this TJ protein appears to participate in M cell transcytosis to intake intestinal antigens[41]. On the other hand, in line with previous research on endurance swimming training in rats[42], both training and exhaustion induced an increase in claudin-2 expression, which, together with claudin-1 and claudin-3, is essential to form the TJ seal[30]. Nevertheless, claudin-2 is a pore-forming claudin[43] and its upregulation has been associated with an undergoing intestinal inflammatory process that contributes to leak-flux diarrhoea[44], which is a frequently reported symptom in endurance athletes[11,31]. Therefore, such increase in claudin-2 could be partially responsible for these gastrointestinal symptoms. Zonula occludens-1 was also upregulated after performing the final additional exhaustion test, in agreement with other authors[39,40,42], while zonula occludens-2 was downregulated. Despite all these changes in TJ proteins' gene expression, we did not observe a marked effect of exercise on intestinal permeability assessed by a1AT concentration in GW, but only a trend of increasing permeability after performing the additional final exhaustion test. Nevertheless, a previous study carried out in trained men found normal levels of faecal a1AT while zonulin's were slightly above normal[45]. Whereas alpha-1-antitrypsin function as a marker of increased intestinal permeability is based on its high concentration in serum and its extravasation into the gut when the epithelial barrier function is impaired, zonulin plays a specific physiological role in disassembling intercellular TJ and altering the paracellular transport of fluids, ions, macromolecules and leukocytes[46]. For that reason, zonulin has recently become one of the most valid surrogate markers to estimate intestinal permeability[31]. In this regard, a recent study compares the intestinal permeability assessment with the traditional assay based on the differential sugar absorption method (lactulose/mannitol test) and zonulin concentration in stool; both were higher in professional athletes than in healthy non-athletes but no significant association between them was found[47]. Therefore, although a growing number of studies using both traditional permeability assays and biomarkers of intestinal inflammation quantification conclude an increased gut permeability after performing intensive exercise[11,48], further studies may clarify the remaining controversies of these results and find the most accurate method to monitor this variable.

With regard to the effects of exercise on the MALT, the majority of exercise studies focused on salivary secretory IgA (SIgA) as a marker of MALT functionality[6,49]. It has been reported that intensive exercise and overtraining produces, in most studies, a decline in salivary SIgA levels[49,50]. The SIgA decrease reported in these studies has been attributed to a reduction in the pIgR expression, an essential receptor involved in the IgA transcytosis across the epithelial cells[52], which could be due to either prolonged sympathetic nervous system overactivation and elevated cortisol concentration[50] or indirectly mediated by cytokines induced by exercise[52]. In agreement with these previous studies[49,50], we found a reduced IgA content in the whole submaxillary salivary gland tissue, that was not counteracted by higher IgM levels[2], which has been reported to be both reduced[56] and increased[57] after performing intensive exercise. Although IgA content in salivary glands is not the same that IgA content in the saliva, it indicates the ability to synthetize this mucosal antibody which will be later submitted to transcytosis and exocytosis, throughout pIgR, to the ducts and eventually will be found in the saliva. Regarding another

mucosal compartment, we found no changes either in the pIgR expression in the wall of small intestine and in the IgA secreted in the intestinal lumen measured in GW. Therefore, further studies must clarify how exercise particularly affects salivary glands and the IgA synthesis, exocytosis and transcytosis therein.

On the other hand, other alterations by the intensive training and the possible overtraining were found in molecules expressed in the GALT. Thus, there was a reduction in RAR-α gene expression due to exhaustion (as observed immediately after the exhaustion test) and an inverse correlation between TGF-β levels and the time supported in the additional final exhaustion test (found 24 h later), meaning that those rats running longer had lower TGF-β expression. Both RAR-α and TGF-β are involved in IgA+ B cell differentiation and gut homing, and their decreased expression after exhaustion could be translated in a lower concentration of intestinal IgA later, as we observed 24 h after exhaustion without reaching significance.

At caecal level, intensive training tended to decrease the caecal IgA content but the final exhaustion test raised it. This increase could be partially due to changes in the caecal microbiota induced by the exhaustion that might involve the release of IgA previously coated to bacteria. In fact, although benefits of moderate exercise in the gut microbiota have been reported[8,57], there is still limited knowledge regarding exhausting exercise-induced microbiota alterations. In this context, whereas some authors reported that high-intensity interval training could overcome some of the detrimental effects in the gut microbiota caused by high-fat diet-induced obesity in mice[58], others have described that healthy mice submitted to a 4-week intensive swimming training hosted a less diverse microbiota[59]. Thus, further studies should be carried out to clarify the effects of intensive exercise and exhaustion in the gut microbiota composition and functionality, as well as the changes induced by exhaustion in the proportion of bacteria coated to IgA.

To take a more in-depth look into understanding exercise-induced GALT alterations, changes in the composition and functionality of mesenteric lymph node lymphocytes were assessed. Neither training nor exhaustion modified the proportion of the minority lymphocyte populations NK, Tγδ and Treg cells. However, we found an inverse correlation between the time supported in the final exhaustion test and the percentage of MLN Tγδ cells in just exhausted rats, probably due to cell apoptosis, in agreement with previous studies in which a decrease in this lymphocyte population in both blood and spleen was observed[23]. Treg cells are an essential subset in the maintenance of immune homeostasis and tolerance and their proportion was also inversely correlated with the performance in exhausted animals. Previous studies found that, in contrast to moderate exercise training[60], intensive training could decrease the number of circulatory Treg in both animal models[61] and marathon runners[62]. This decrease was attributed at least partially to the higher cortisol production observed after exhaustion as we previously observed in similarly trained rats[23], since an in vitro study demonstrated that dexamethasone (synthetic glucocorticoid) exposure for 24 h decreases FoxP3+ expression in peripheral blood mononuclear cells[63]. On the other hand, unlike what has been previously described in spleen and blood[23], MLN NK cell proportion was not modified by either intensive training or the final exhaustion.

Regarding the predominant lymphocyte populations, the intensive training programme and possible overtraining increased the proportion of Tαβ+ lymphocytes -and above all, Th cells since the Tc proportion was significantly lower—while it decreased that of B cells. Nevertheless, after the final exhaustion test, both B and T cell proportions were similar to that in sedentary rats. Other studies have reported a reduced T cell proportion in mouse submandibular lymph nodes after carrying out an intensive treadmill run test[24]. It could be hypothesized that with exhaustion, MLN Th cells move to the blood thus decreasing (normalizing) their proportion in the MLN; however, we have previously described a reduction in both blood Th and Tc cell proportions due to exhaustion[23]. In this case, these changes might be explained by a redistribution of lymphocytes within other lymphoid and non-lymphoid organs[25] and the lymphocyte apoptosis[64,65] induced by the release of catecholamines and glucocorticoids due to the intensive exercise stress. In this regard, a previous study in animals with a similar training program showed higher cortisol levels [23,26].

Apart from the changes in MLN T and B lymphocyte proportions, intensive training and exhausting exercise also modified their functionality. In the case of T lymphocytes, the intensive training programme, possibly inducing overtraining, decreased their proliferation capacity. Previous research has already reported the reduced lymphocyte proliferation capacity induced by intensive training[66] and even acute bouts of exercise of a wide range of intensities[67]. This decrease could be partially due to the lymphopaenia and the higher cortisol levels found in rats which were similarly trained[23,26], since glucocorticoids' immunosuppressant properties target both cell trafficking and proliferation capacity[67,68]. On the other hand, the well-characterized exhausting exercise-induced acute lymphocytosis[49,50], which we previously reported[26], together with the increase in IL-2 secretion by MLN lymphocytes observed in the current study and by other authors[69,70], may explain the sudden increase in proliferation right after performing the final exhaustion test.

With regard to other cytokines released by stimulated MLNLs, we observed an increase in IFN-γ production by lymphocytes from trained animals, which was even higher in rats who performed the final exhaustion test. The IFN-γ/IL-4 ratio (Th1/Th2 balance) was also higher in exhausted animals, showing a Th1 bias in MLNs, probably evidencing a local inflammatory process. Concerning IL-10 secretion, it positively correlated with the performance achieved in the final exhaustion test, again evidencing an intensity and/or running length-dependent exercise-induced immune disruption. The exhaustion-induced cortisol increase we previously reported[23] could explain this association, since an in vitro study reported an increase in IL-10 expression in peripheral blood mononuclear cells after 24 h culture with dexamethasone[63].

Finally, in order to assess the functionality of MLN B lymphocytes, IgA, IgM and IgG concentrations in cell supernatants were quantified. There was a higher MLN production of IgG due to both the intensive training and exhaustion, although these levels were lower 24 h later. There were no changes in IgA and IgM production due to exercise. These findings are in line with previously reported results in serum[23]. We hypothesized that, as previously reported in spleen[23], MLN could be sensitive to the repetitive stress induced by the intensive training programme, which might upregulate the glucocorticoids and adrenergic receptors[71] and this, in addition to the

higher cortisol levels observed in similarly exercised animals[23], may explain the higher in vitro production of IgG by MLN lymphocytes.

In summary, intensive training for 5 weeks in female Wistar rats, followed or not by an additional exhaustion test, appears to have caused an overtraining condition which modified both mucosal immunity and the intestinal epithelial barrier integrity. In particular, the intensive training programme decreased the salivary IgA concentration, impaired the claudins and occludin gene expression in the small intestine and altered the mesenteric lymph node lymphocyte composition while decreasing their proliferation capacity and increasing their IFN-γ secretion. The final exhaustion enhanced the caecal production of IgA while it impaired the zonula occludens expression and enhanced the IL-2 and IFN-γ secretion by mesenteric lymph node lymphocytes. These findings could partially explain the decline of the mucosal immunity and the gastrointestinal symptomatology induced by intensive exercise and in overtrained athletes. Further research might clarify whether a more intensive or longer exercise training can exacerbate the observed alterations and search for accurate nutritional strategies to counteract and even prevent them.

Methods

Animals. Female Wistar rats (3 week-old at arrival) were provided by Envigo (Huntingdon, United Kingdom) and were maintained at the animal facility of the Faculty of Biology (University of Barcelona). Female rats were used because previous studies reported a better adaptation to treadmill running than male rats[34,73] while the impact of exercise on immunological variables was not influenced by gender[73]. The animals were kept under controlled conditions of temperature and humidity, in a 12 h/12 h light/dark cycle. Animals (2–3 per cage) were given ad libitum access to food (Teklad Global 14% Protein Rodent Maintenance Diet, Teklad, Madison, WI, USA) and water. Body weight (BW) and food intake were monitored throughout the study. Animal procedures were approved both by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 464/16) and the Catalonia Government (DAAM 9257). The number of animals was established according to the minimum required for providing statistically significant differences among groups, using the Appraising Project Office's programme from the Universidad Miguel Hernández de Elche (Alicante, Spain). Moreover, the number of rats in each group was adjusted following the University Ethical Committee guidelines and applying the three Rs rule for experimenting in animals. All methods were carried out in accordance with relevant guidelines and regulations.

Training programme. After a 2-week adaptation period, rats were submitted to a high-intensity exercise training (n = 24) or remained as a sedentary control group (n = 8; SED). A high-intensity training was induced in rats by running in a treadmill (LE8700, Panlab, Harvard, USA, and Exer3/6 treadmill Columbus, Ohio, USA) 5 days per week for 5 weeks, involving two exhaustion tests per week, as previously reported[23,26]. Briefly, every Monday and Friday, the exercised group carried out an exhaustion test, which consisted of running 15 min at 60% of the maximum speed average achieved in the previous Monday's exhaustion test (the speed of the first Monday's exhaustion test was 30 m × min^{-1}), and from then on, the speed was progressively increased until exhaustion. On Tuesday, Wednesday and Thursday, rats trained for 20, 25 and 30 min, respectively, at 60% of the maximum speed average achieved in the previous Monday's exhaustion test. At the end of the training period, runner animals were distributed into 3 groups (n = 8) with a similar ability to run: trained group (T, whose samples were obtained 24 h after a regular training), exhausted group (TE, whose samples were obtained immediately after an additional final exhaustion test) and 24 h post-exhaustion group (TE24, whose samples were obtained 24 h after the additional final exhaustion test). The SED group was exposed to the same conditions of maintenance and isolation stress as runner rats. As a positive reinforcement, both runner and SED rats received at the end of training or isolation, a 50% solution of condensed milk (100 µL/100 g BW).

Sample collection and processing. At the end of the study, animals were anaesthetized by intramuscular injection of ketamine (Merial Laboratories S.A. Barcelona, Spain) and xylazine (Bayer A.G., Leverkusen, Germany) (90 mg × kg^{-1} and 10 mg × kg^{-1}, respectively) and exsanguinated. The MLN, small intestine, SMG and CC were collected.

The lymphocytes from MLN were isolated in aseptic conditions by passing the tissue through a 40 µm sterile mesh cell strainer (Thermo Fisher Scientific, Barcelona, Spain), as previously detailed[72]. MLNL counting and viability were determined by a Countess Automated Cell Counter (Invitrogen, Thermo Fisher Scientific).

A 0.5 cm portion of the middle part of the small intestine was immediately kept in RNAlater (Ambion, Life Technologies, Austin, TX, USA) and stored at − 20 °C until the determination of gene expression of some molecules by Real-Time Polymerase Chain Reaction (RT-PCR). The distal part of the small intestine was used to obtain GW, as established previously in our laboratory[73]. Briefly, it was flushed with cold PBS (pH 7.2) to remove faecal content, opened lengthwise, cut into 1–2 cm pieces, weighed and incubated with 3 mL of PBS for 10 min in a shaker at 37 °C (55 shakings × min^{-1}). After centrifugation (538 g, 4 °C, 10 min), supernatants were collected and stored at − 20 °C until a1AT and IgA quantification.

SMG and CC homogenates were obtained using a tissue homogenizer (Polytron, Kinematica, Lucerne, Switzerland and Pellet Pestle Cordless Motor, Kimble, Meiningen, Germany, respectively), as described in previous studies, and kept at − 20 °C until IgA quantification[74].

MLNL phenotypic analysis. MLNLs (5×10^5 cells) were extracellularly and intracellularly stained by using mouse anti-rat monoclonal antibodies (mAb) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-a protein (PercP), allophycocyanin (APC) and brilliant-violet 421 (BV421), as previously described[72]. The following fluorochrome-conjugated mAb antibodies were used: FITC-TCRαβ, FITC-

CD8β, FITC-CD25, PE-CD161a, PE-TCRγδ, PE-CD4, PerCP-CD8α, APC-CD4, and BV421-CD45RA (BD Biosciences, Madrid, Spain) and APC-FoxP3 (eBioscience, Frankfurt, Germany). For extracellular staining, MLNL were incubated with saturating amounts of mAb in PBS containing 2% FBS and 0.1% NaN_3 (darkness, 4 °C, 20 min). For intracellular staining, cells previously labelled extracellularly with anti-CD4-PE and anti-CD25-FITC mAb were treated with Foxp3 fixation/permeabilization kit (eBioscience). Then, intracellular staining with anti-Foxp3-APC mAb was carried out (darkness, 4 °C, 30 min), as described in previous studies[75]. All stained cells were fixed with 0.5% p-formaldehyde and stored at 4 °C in darkness until analysis by flow cytometry. A negative control staining without any mAb antibody and a staining control for each mAb were included. Analyses were performed using a Gallios Cytometer (Beckman Coulter, Miami, FL, USA) in the Flow Cytometry Unit of the Scientific and Technological Centres of the University of Barcelona (CCiT-UB) and by Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA). Changes in lymphocyte phenotype by exercise are represented considering the SED group mean value as 1, therefore, all values are expressed as a fold change of the mean value with respect to the SED group.

MLNL stimulation and proliferation. MLNL (10^5 cells/well) were incubated in quadruplicate in 96-well plates (TPP, Sigma-Aldrich, Madrid, Spain) and stimulated or not with ConA (5 µg × mL^{-1}, Sigma-Aldrich) for 48 h. T cell proliferation was quantified using a BrdU Cell Proliferation Assay kit (MerckMillipore, Darmstadt, Germany), according to manufacturer's instructions. The proliferation rate was calculated by dividing the optical density of ConA stimulated cells with the optical density of non-stimulated cells. Changes in MLNL proliferative capacity by exercise are represented considering the SED group mean value as 1.

Moreover, after 48 h of incubation, supernatants from ConA-stimulated and non-stimulated conditions were collected and stored at − 80 °C in order to evaluate the cytokine production. Supernatants from non-stimulated conditions were also used to quantify IgA, IgM and IgG.

Cytokine quantification. The production of IFN-γ, IL-2, IL-4 and IL-10 was determined in MLNL supernatants by ProcartaPlex Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, USA), according to the manufacturer's protocol. Data were acquired by MAGPIX Cytometer (Affymetrix) in the CCiT-UB and analysed by ProcartaPlex Analyst v1.0 software (Affymetrix). The lower limits of detection were as follows: 3.34 pg × mL^{-1} for IFN-γ; 1.82 pg × mL^{-1} for IL-2; 0.62 pg × mL^{-1} for IL-4; 6.01 pg × mL^{-1} for IL-10. The ratio of mean fluorescence intensity (MFI) obtained from cells supernatants under ConA stimulation with respect to the non-stimulated condition was calculated. Changes in MLNL cytokine profile by exercise are expressed considering the SED group mean value as 1.

Immunoglobulin quantification. The concentrations of IgG, IgM and IgA in MLNL culture supernatants, GW, SMG or CC homogenates were quantified by a sandwich ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA). After assay development as previously described[76], absorbance was measured on a microplate photometer (Labsystems Multiskan, Helsinki, Finland) and data were analysed by Ascent v.2.6 software (Thermo Fisher Scientific, S.L.U, Barcelona, Spain) according to the respective standard curves. The immunoglobulin content in SMG and CC was normalized by total protein concentration which was measured using the Pierce-660 nm ready-to-use Protein Assay Reagent (Thermo Fisher Scientific). Changes in each Ig concentration are expressed considering the SED group mean value as 1.

Gene expression in small intestine by real-time polymerase chain reaction (RT-PCR). The intestinal expression of occludin, claudin-2, claudin-4, ZO-1, ZO-2, pIgR, TGF-β1, RAR-α and CCL25 was assessed in the small intestine. This tissue, kept in RNA later, was homogenized in a lysing matrix tube (MP Biomedicals, Illkirch, France) by a FastPrep-24 instrument (MP Biomedicals) for 30 s. Intestinal RNA was then isolated with the RNeasy mini kit (Qiagen, Madrid, Spain) following the manufacturer's instructions. RNA quantification and purity were assessed using a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, USA). RNA was reverse-transcribed in a thermal cycler (PTC-100 Programmable Thermal Controller, BioRad, Hercules, CA, USA) using TaqMan Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany) in order to obtain the corresponding cDNA.

The RT-PCR was carried out in duplicate for each sample using the ABI Prism 7900 HT quantitative RT-PCR system (AB) with the following specific PCR TaqMan primers and probes (AB): Ocln (Rn00580064_m1, Inventoried, I); Cldn2 (Rn02063575_s1, I); Cldn4 (Rn01196224_s1, I); ZO1 (Rn02116071_s1, I); ZO2 (Rn01501483_m1, I); Pigr (Rn00562362_m1, I); Tgfb1 (Rn00572010_m1, I); Rara (Rn00580551_m1,I); and Ccl25 (Rn01403352_m1, I). SDS version 2.4 software (AB) was used to assess the gene expression. The gene expression of the target genes was normalized with respect to the housekeeping genes *Gusb* (β-glucuronidase, Rn00566655_m1, I) or *β-actin* (Rn00667869_m1, I), depending on their expression level, using the $2^{-\Delta\Delta Ct}$ method[77]. For *Cldn2* and *ZO-1* the gene expression of *Gusb* was used as housekeeping gene, whereas for the rest of the genes, *β-actin* expression was used. Changes in small intestine gene expression are expressed considering the SED group mean value as 1.

Small intestine permeability. The concentration of a1AT in GW was assessed as a marker of intestinal protein loss and mucosal permeability. The quantification was performed with the ELISA Kit for rat a1AT (Cloud-CloneCorp., Houston, TX, USA) following the manufacturer's instructions. Absorbance was measured on a microplate photometer (LabSystems Multiskan) and data were interpolated by Ascent v.2.6 software (Thermo Fisher Scientific) according to the standard, which ranged from 500 to 7.8 ng × mL^{-1}. Changes in GW a1AT concentration are expressed considering the SED group mean value as 1.

Statistical analysis. Statistical analysis of the data was carried out using IBM Social Sciences Software Program (SPSS, version 26.0, Chicago, IL, USA). Variance equality and normality of the data was tested by Levene's and Shapiro–Wilk's test, respectively. A one-way ANOVA test was applied and, if significant differences were detected, Tukey's post hoc test was performed. Kruskal–Wallis test was used when results were neither equally nor normally distributed, followed by Mann–Whitney U test in the case of significant differences among groups. The Spearman correlation coefficient was used to assess correlations between the variables analysed and the performance achieved. When comparing variables from two groups throughout the study, the repeated measures ANOVA test was used to assess whether there was a significant interaction between time (day of study) and exercise condition (SED or runner). Once we confirmed there was a significant interaction ($p<0.05$ in Greenhouse–Geisser test, Huynh–Feldt test and Lower-bound test), we performed the unpaired Student's t test to detect between which days of study the differences between SED and runner rats were statistically significant. To compare variables during the study (e.g., maximum time lasted in the exhaustion tests), a repeated-measures ANOVA was applied followed by paired Student's t test. Significant differences were considered when $p<0.05$.

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Author contributions
M.C.-B., M.C. and F.J.P.–C. conceived and designed the experiments; P.R.-I., S.E.-A., and M.M.-C. performed the experiments, P.R.-I. analysed the data and wrote the paper, and M.M.-C., M.C. and F.J.P.–C. reviewed the manuscript. All authors have read, reviewed and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to M.C. or F.J.P.-C.

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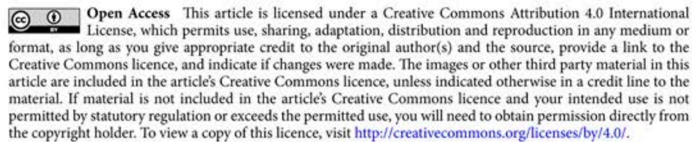

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ARTICLE 3

"Protective effect of a cocoa-enriched diet on oxidative stress induced by intensive acute exercise in rats"

Patricia Ruiz-Iglesias, Malén Massot-Cladera, Maria J. Rodríguez-Lagunas, Àngels Franch, Mariona Camps-Bossacoma, Francisco J. Pérez-Cano, Margarida Castell.

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ABSTRACT

Aim: To establish the influence of cocoa and cocoa fibre on the oxidative stress and systemic immune alterations induced by a single session of exhausting exercise in rats.

Methods: Wistar rats were fed either a standard diet, a diet containing 5% cocoa fibre or a diet with 10% cocoa. After 4 weeks, half of the rats remained sedentary and the second half run in a treadmill until exhaustion. Sixteen hours later, blood samples and peritoneal macrophages were obtained to assess the systemic immune status and the production of reactive oxygen species (ROS).

Results: The increase in ROS production induced by a single session of exhausting exercise was prevented by both cocoa and cocoa fibre-enriched diets. Nevertheless, none of these diets avoided the differential changes in circulating leukocytes and the decrease in serum IgG levels induced by acute exercise.

Conclusion: Both cocoa and cocoa fibre diets protect against the oxidative stress induced by a single session of exhausting exercise, nevertheless, these diets did not avoid the changes induced by exercise on immune function.

antioxidants

Article

Protective Effect of a Cocoa-Enriched Diet on Oxidative Stress Induced by Intensive Acute Exercise in Rats

Patricia Ruiz-Iglesias [1,2], Malén Massot-Cladera [1,2], Maria J. Rodríguez-Lagunas [1,2], Àngels Franch [1,2], Mariona Camps-Bossacoma [1,2], Francisco J. Pérez-Cano [1,2,*] and Margarida Castell [1,2,3,*]

[1] Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), 08028 Barcelona, Spain; patriciaruiz@ub.edu (P.R.-I.); malen.massot@ub.edu (M.M.-C.); mjrodriguez@ub.edu (M.J.R.-L.); angelsfranch@ub.edu (À.F.); marionacamps@ub.edu (M.C.-B.)
[2] Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), 08921 Santa Coloma de Gramenet, Spain
[3] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain
* Correspondence: franciscoperez@ub.edu (F.J.P.-C.); margaridacastell@ub.edu (M.C.); Tel.: +34-93-402-45-05 (F.J.P.-C. & M.C.)

Abstract: Intensive acute exercise can induce oxidative stress, leading to muscle damage and immune function impairment. Cocoa diet could prevent this oxidative stress and its consequences on immunity. Our aim was to assess the effect of a cocoa-enriched diet on the reactive oxygen species (ROS) production by peritoneal macrophages, blood immunoglobulin (Ig) levels, leukocyte counts, and the physical performance of rats submitted to an intensive acute exercise, as well as to elucidate the involvement of cocoa fiber in such effects. For this purpose, Wistar rats were fed either a standard diet, i.e., a diet containing 10% cocoa (C10), or a diet containing 5% cocoa fiber (CF) for 25 days. Then, half of the rats of each diet ran on a treadmill until exhaustion, and 16 h later, the samples were obtained. Both C10 and CF diets significantly prevented the increase in ROS production. However, neither the cocoa diet or the cocoa fiber-enriched diet prevented the decrease in serum IgG induced by acute exercise. Therefore, although the cocoa-enriched diet was able to prevent the excessive oxidative stress induced by intensive exercise, this was not enough to avoid the immune function impairment due to exercise.

Keywords: acute exercise; fiber; flavanols; immunoglobulin; leukocytes; lymphocytes; oxidative stress; polyphenols; ROS

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1. Introduction

Cocoa beans are the seeds of the fruit from the *Theobroma cacao* L. tree. After fermentation, drying, and subsequent processing, cocoa powder is obtained. Over the last centuries, some medicinal properties have been attributed to cocoa [1], but it has only been in recent years that cocoa has become a subject of increasing interest because of its several beneficial effects on human health, including as an antioxidant [2], a blood pressure regulator [3] and its immunomodulatory [4] and anti-inflammatory [5] properties. These effects are mainly attributed to its polyphenol content, which mostly consists of flavanols and their polymers. More specifically, cocoa polyphenol content comprises about 58% of proanthocyanidins, 37% of catechins, and 4% of anthocyanidins [6]. The predominant catechin monomer found in cocoa is (−)-epicatechin [6], and given its high abundance, it is believed that it could play an essential role in the beneficial effects of cocoa. For this reason, several authors have focused their attention on the isolated effect of this catechin on exercise performance [7], muscle oxygenation [8], and cytokine production [9], among others [10]. However, it is important to consider that cocoa also contains other bioactive compounds, such as dietary

fiber and methylxanthines, which may contribute to cocoa's beneficial effects on health and particularly on the immune system [11,12].

Acute intensive exercise often triggers an imbalance between endogenous oxidants and antioxidants, in favor of the oxidants, a phenomenon that is commonly known as oxidative stress [13]. Reactive oxygen species (ROS) are oxygen-containing reactive molecules and free radicals generated as a result of molecular oxygen reduction during normal cellular metabolism processes [14]. They are involved in many physiological processes, such as protein phosphorylation, transcriptional factors activation, immune system signaling, and apoptosis [14]. However, an excessive accumulation of extracellular free radicals impairs the immune system functionality, leading to a systemic inflammatory status [15] that could contribute to the development of a great number of pathologies, such as autoimmune, metabolic and neurodegenerative diseases, or even cancer [16]. In the athletic area, muscle damage, physical fatigue, and an impaired exercise performance have also been associated with an overproduction of ROS induced by intensive exercise [17]. For this reason, in this field, supplementation with exogenous antioxidants to counteract these phenomena and, consequently, improve exercise performance [17] is of increasing interest. Moreover, the prescription of exercise-based rehabilitation programs in the clinical practice has grown lately. The mentioned programs are considered a safe intervention [18] to enhance endogenous antioxidant systems through sirtuin 1 (SIRT1) activation [19], among other beneficial effects; however, exogenous antioxidant supplementation may be a useful complementary therapy in patients with diseases associated with oxidative stress [20].

Exogenous antioxidants can be obtained through most fruits and vegetables, but the amount of them present in a standard diet may be insufficient to support intensive exercise demands. Hence, some commercial supplements containing polyphenols and/or other molecules with antioxidant properties, such as vitamins E, C, and A and some minerals, are available [17]. Polyphenols exert their antioxidant capacity mainly through the direct neutralization of free radicals and chelating metals such as Fe^{2+} and Cu^{+} [21]. However, other relevant mechanisms have been reported, such as the stimulation of mitochondrial biogenesis through the activation of SIRT1 [19,22] and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [23] signaling pathways. Cocoa, due to its polyphenol content, seems a good source of exogenous antioxidants that may be useful for preventing some of the alterations induced by intensive exercise, such as muscular damage [24], immune disruption [25–27], and even the decline in performance, although the available evidence does not support the use of cocoa as an ergogenic aid [28]. Moreover, isolated cocoa fiber, even containing a much lower percentage of polyphenols, has also demonstrated antioxidant properties [29,30], which, together with its beneficial effects in the intestinal compartment [31], may also be a good supplement to avoid the undesirable effects of overly intensive exercise [32].

We previously demonstrated that hesperidin supplementation, which is the main polyphenol found in citrus fruits, was able to prevent the overproduction of ROS induced by exhausting exercise through enhancing the endogenous antioxidant systems of intensively trained rats [33]. Moreover, we recently found the beneficial effects of cocoa, especially from its fiber content, on the cecal microbiota and the mucosal immune system of rats submitted to acute exercise [34]. Here, we aimed to evaluate the effect of a cocoa-enriched diet on the physical performance, the ROS production by peritoneal macrophages, and the systemic immune function of rats submitted to intensive acute exercise, as well as to elucidate the involvement of cocoa fiber in such effects.

2. Materials and Methods

2.1. Animals

Female Wistar rats (four-week-old) were provided by Envigo (Huntingdon, UK) and maintained in the animal facilities of the Faculty of Pharmacy and Food Science at the University of Barcelona. Female rats were used, because they showed a better adaptation to the treadmill and exercise performance than male rats in previous studies from our laboratory [25] and from others [35], whereas the impact of exercise on immunological variables was not

influenced by rat gender [25]. The rats were kept in polycarbonate cages, with four animals per cage, under controlled conditions of temperature and humidity in a 12 h light/12 h dark cycle and had ad libitum access to food and water. The animal procedure was approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEEA/UB ref. 517/18 and DAAM 9257, respectively). All methods were carried out in accordance with relevant guidelines and regulations.

2.2. Nutritional and Exercise Intervention

The nutritional and exercise intervention applied in the current study has already been reported [34]. Briefly, all rats were first familiarized with running on a rodent treadmill (Exer3/6, Columbus, OH, USA) for one week, with the increasing running time and speed. Then, animals performed an exhaustion test (ET) in which, after 10 min running at 18 m/min, the speed was increased every 2 min (by 3 m/min) until rat exhaustion. The end of the test was established, when rats touched the shock grid more than three times. Rats were then homogeneously distributed in six groups according to their running capacity (n = 8/each)—REF/C, REF/R, C10/C, C10/R, CF/C, and CF/R—that received three different isoenergetic diets during 25 days as in previous studies [31]. The R groups would run again after the 25-day diet, whereas the C groups would not.

The reference (REF) groups (i.e., REF/C and REF/R groups) received a standard diet AIN-93M (maintenance diet from the American Institute of Nutrition, Envigo, Huntingdon, UK). The cocoa (C10) groups (i.e., C10/C and C10/R groups) were fed a diet containing 10% defatted cocoa (Idilia Foods S.L., Barcelona, Spain) providing a final proportion of 3.6 g/kg polyphenols, 6.0 g/kg soluble fiber, and 54.0 g/kg insoluble fiber. The cocoa fiber (CF) groups (i.e., CF/C and CF/R groups) were fed a diet containing 5% cocoa fiber powder (Idilia Foods S.L.), which provided 0.4 g/kg of polyphenols, 8.0 g/kg soluble fiber, and 56.0 g/kg insoluble fiber. Thus, the CF diet provided a similar proportion of fiber to the C10 diet but much lower amount of polyphenols, as applied in previous studies [11].

One week prior to the end of the dietary intervention, all rats were again familiarized with running on the treadmill. The final ET, only performed by the R animals, consisted of 15 min running at 18 m/min and then every 2 min with a speed increased by 3 m/min until exhaustion. This ET was carried out in the afternoon, between 5 and 8 p.m.

2.3. Sample Collection

Sixteen hours after the ET of R rats, both R and C rats were euthanized. For this, animals were anesthetized intramuscularly with ketamine (90 mg/kg; Merial Laboratories S.A. Barcelona, Spain) and xylazine (10 mg/kg; Bayer A.G., Leverkusen, Germany). Peritoneal macrophages were immediately collected and used to assess ROS production. Moreover, anticoagulated blood (EDTA-K_3) was obtained from the heart and was immediately analyzed using an automated hematology analyzer (Spincell, MonLab Laboratories, Barcelona, Spain). Other blood samples were used to obtain plasma and serum, which were maintained at −80 or −20 °C until hormone and immunoglobulin (Ig) quantification, respectively. The spleen and heart were also collected and weighed.

2.4. Peritoneal Macrophages Isolation and ROS Production

To collect the peritoneal macrophages, 40 mL of cold, sterile phosphate-buffered saline (PBS, pH 7.2) were injected into the peritoneal cavity for 2 min under massage and immediately collected, as described previously [33,36]. The cell suspension obtained was centrifuged (speed: 538× *g*; duration: 10 min; temperature: 4 °C) and resuspended with cold Roswell Park Memorial Institute (RPMI) media without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL streptomycin–penicillin, and 2 mM L-glutamine (all from Sigma-Aldrich, Madrid, Spain). After counting the macrophages using a Spincell hematology analyzer (MonLab Laboratories), 10^4 cells/well were plated on a black 96-well plate (Thermo Fisher Scientific, Barcelona, Spain) and incubated overnight to allow their attachment to the plate. On the next day, cells were

washed with warm RPMI media, and the ROS production was assessed after incubation for 30 min with 20 μM of reduced 2′,7′-dichlorofluorescein diacetate probe (H2DCF-DA; Invitrogen, Paisley, UK) in order to oxidize the H2DCF-DA to a fluorescent compound (2′,7′-dichlorofluorescein) [33,36]. Then, 0.5 mM of H_2O_2 was added to the plate, and the fluorescence was measured once every 15 min for 2 h by the fluorimeter Modulus Microplate Multimode Reader (Turner BioSystems, Sunnyvale, CA, USA).

2.5. Plasma Cortisol and Noradrenaline (NA) Concentration

The quantification of cortisol in plasma was performed with the DetectX® Cortisol competitive enzyme-linked immunosorbent assay (ELISA; Arbor Assays, Ann. Arbor, MI, USA) following the manufacturer's instructions. Plasma NA concentration was quantified using an NA/norepinephrine (NA/NE) competitive ELISA Kit (Elabscience, Houston, TX, USA) following the manufacturer's instructions. In both cases, absorbance was measured on a microplate photometer (Labsystems Multiskan), and data were interpolated by Ascent v.2.6 software (Thermo Fisher Scientific) according to the respective standard.

2.6. Serum Immunoglobulins Concentration

The concentrations of IgG, IgM, and IgA in sera were determined by a sandwich ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA), as previously detailed [37]. Results were analyzed in a microplate photometer, as mentioned before, and data were interpolated by the same software.

2.7. Statistical Analysis

The analysis of the data was performed using IBM Social Sciences Software Program (SPSS, version 26.0; Chicago, IL, USA). Once the normality and equality of the data were confirmed by a Shapiro–Wilk and Levene test, respectively, a two-way ANOVA test was applied. If significant differences were detected, Tukey's post hoc test was carried out. When the data were neither equally nor normally distributed, a Kruskal–Wallis test followed by a Mann–Whitney U test was applied. Significant differences were considered for $p < 0.05$.

3. Results

3.1. Exercise Performance

The time lasted in the final ET (Figure 1a) was monitored as a measure of exercise performance. This time was about 30 min in control rats, and the diets enriched in cocoa or cocoa fiber did not statistically modify it.

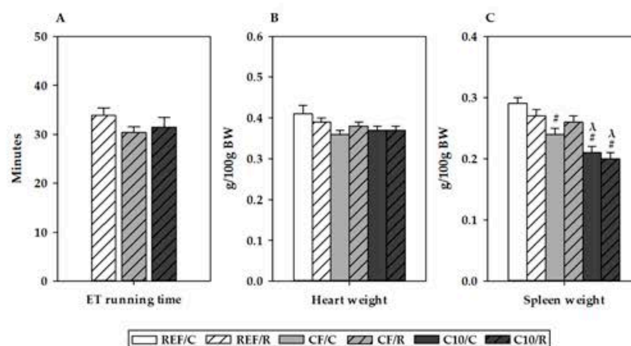

Figure 1. Running time during the final exhaustion test for running (R) rats (**A**) and relative heart (**B**) and spleen (**C**) weights for all groups. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10%

cocoa-enriched diet. The control (C) groups are represented by smooth bars, and the R groups are represented by striped bars. Data are expressed as mean ± standard error of the mean (SEM) (n = 8). Statistical differences (a Kruskal–Wallis test followed by a Mann–Whitney U test): $^{\#}$ $p < 0.05$ vs. the same exercise condition in the REF diet; $^{\lambda}$ $p < 0.05$ vs. the same exercise condition in the CF diet.

3.2. Organ Weight and Plasma Cortisol and NA Concentrations

Sixteen hours after performing the final ET, tissue and blood samples were obtained. Acute exercise did not modify the weight of the heart or spleen (Figure 1b,c). Likewise, the heart weight was not influenced by the C10 or CF diets. However, the intake of the C10 diet lowered the relative spleen weight in C10/C and C10/R animals (p = 0.001), whereas the CF diet decreased it in sedentary CF/C rats (p = 0.007).

On the other hand, 16 h after the final ET, REF/R rats showed a lower concentration of plasma cortisol than REF/C animals (about a 40% decrease, p = 0.035; Figure 2a). The C10 diet decreased the plasma cortisol concentration in C10/C animals, with this change only being significant when it was compared with in the CF/C group (p = 0.016). Neither the acute exercise nor the experimental diets induced statistically significant changes in plasma NA (Figure 2b).

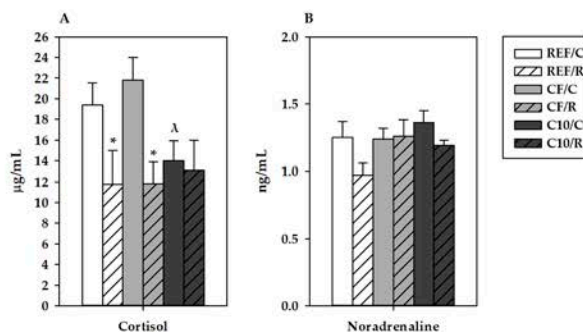

Figure 2. Plasma cortisol (**A**) and noradrenaline (**B**) concentrations 16 h after performing the final exhaustion test. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. The control (C) groups are represented by smooth bars, and the runner (R) groups are represented by striped bars. Data are expressed as mean ± SEM (n = 8). Statistical differences (a Kruskal–Wallis test followed by a Mann–Whitney U test): * $p < 0.05$ vs. the C group in the same diet; $^{\lambda}$ $p < 0.05$ vs. the same exercise condition in the CF diet.

3.3. Hemograme

Blood leukocyte counts were similar in animals fed standard diet and in animals fed CF and C10 diets after 16 h of a single bout of intensive exercise (Figure 3a). With regard to differential leukocytes (Figure 3b), acute exercise in animals following REF and CF diets, but not C10 diet, induced a decrease in the proportion of lymphocytes, which achieved statistical significance only for the CF group (p = 0.009, CF/C vs. CF/R; p = 0.07, REF/C vs. REF/R). Reciprocally, there was an increase in the percentage of granulocytes by acute exercise in animals fed REF and CF diets (p = 0.009, CF/C vs. CF/R; p = 0.05, REF/C vs. REF/R). The percentage of monocytes was not modified either by acute exercise or by the intake of the experimental diets.

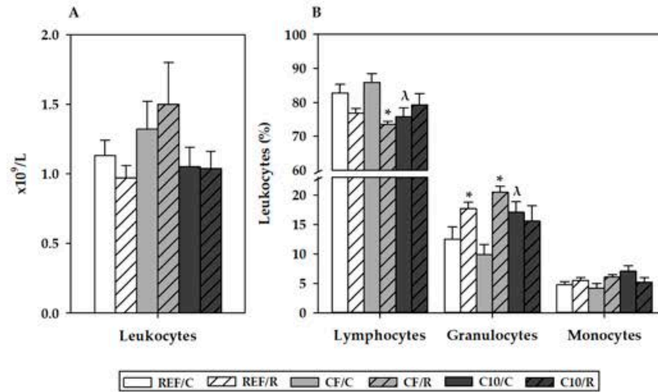

Figure 3. Blood counts of leukocytes (**A**) and proportions of lymphocytes, granulocytes, and monocytes (**B**) 16 h after performing the final exhaustion test. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. The control (C) groups are represented by smooth bars, and the runner (R) groups are represented by striped bars. Data are expressed as mean ± SEM (n = 8). Statistical differences (a Kruskal–Wallis test followed by a Mann–Whitney U test): * $p < 0.05$ vs. the C group in the same diet; $^{\lambda}$ $p < 0.05$ vs. the same exercise condition in the CF diet.

With regard to results related to red blood cells (Figure 4), erythrocyte counts, hemoglobin (HGB) concentration, and hematocrit (HCT) were not affected after 16 h of an acute exercise. However, there was an increase in the erythrocyte counts and HCT after both C10 and CF diets ($p < 0.05$) and in the HGB concentration after following the C10 diet ($p = 0.001$).

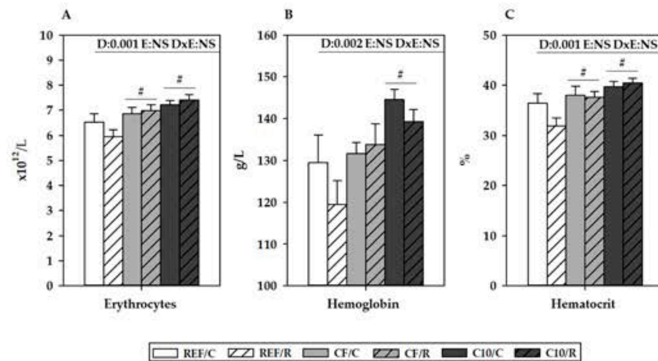

Figure 4. Blood erythrocyte counts (**A**), hemoglobin concentration (**B**), and hematocrit (**C**) 16 h after performing the final exhaustion test. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. The control (C) groups are represented by smooth bars, and the runner (R) groups are represented by striped bars. Data are expressed as mean ± SEM (n = 8). Statistical differences (two-way ANOVA followed by Tukey's post-hoc test): # $p < 0.05$ vs. the REF diet.

3.4. ROS Production by Peritoneal Macrophages

The ROS production by peritoneal macrophages in runner rats fed REF diet was higher than that of REF/C rats ($p = 0.037$) (Figure 5). This exercise-induced increase was prevented by both experimental diets.

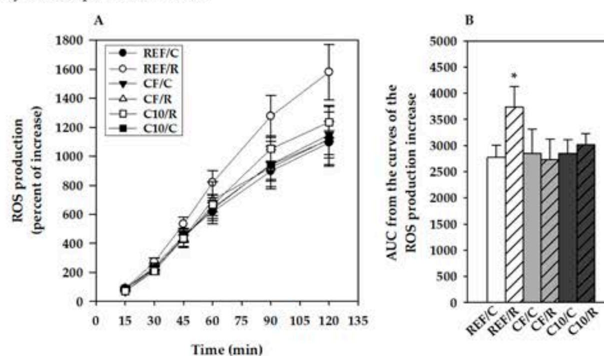

Figure 5. Reactive oxygen species (ROS) production by peritoneal macrophages 16 h after performing the final exhausion test. (**A**) Kynetics of ROS production for 2 h; (**B**) Area under the curve (AUC) between 15–120 min. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet; C, control groups; R, runner groups. Data are expressed as mean ± SEM (n = 8). Statistical differences (a Kruskal–Wallis test followed by a Mann–Whitney U test): * $p < 0.05$ vs. the C group in the same diet.

3.5. Serum Immunoglobulins

The serum concentrations of IgG, IgM, and IgA were determined in samples obtained 16 h after the final ET (Figure 6). Acute exercise induced a 50% decrease in IgG ($p = 0.003$), whereas it did not affect the levels of IgM and IgA.

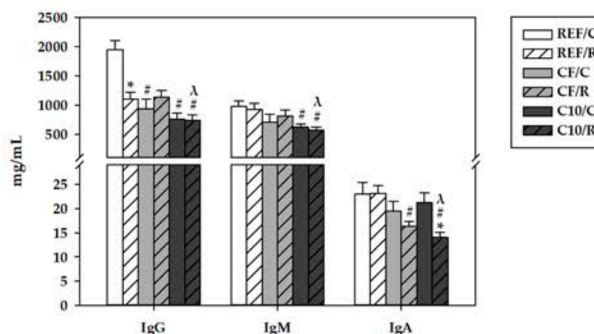

Figure 6. Serum concentrations of IgG, IgM and IgA 16 h after performing the final exhaustion test. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. The control (C) groups are represented by smooth bars, and the runner (R) groups are represented by striped bars. Data are expressed as mean ± SEM (n = 8). Statistical differences (a Kruskal–Wallis test followed by a Mann–Whitney U test): * $p < 0.05$ vs. the C group in the same diet; $^{\#}$ $p < 0.05$ vs. the same exercise condition in the REF diet; $^{\lambda}$ $p < 0.05$ vs. the same exercise condition in the CF diet.

With regard to the nutritional intervention, both C10 and CF diets lowered the serum IgG levels in the sedentary C groups ($p < 0.005$), and they were not influenced by exercise. The C10 diet also induced a decrease in serum IgM, independently of the exercise conditions ($p < 0.05$). On the other hand, acute exercise decreased the serum concentrations of IgA in rats fed C10 and CF diets ($p < 0.005$ vs. REF/R).

4. Discussion

Previous preclinical studies have reported the antioxidant, immunomodulatory, and anti-inflammatory effects of a cocoa-enriched diet in preclinical models of immune-mediated diseases such as arthritis [36,38], allergy [39,40], and inflammation [41]. Moreover, dietary interventions with cocoa fiber have shown beneficial effects, not only in the intestinal compartment [31], but also in the oxidative stress induced by an inflammatory state, such as metabolic syndrome [29]. However, to our knowledge, there are no preclinical studies focused on the beneficial properties of cocoa on the oxidative stress induced by intensive exercise. Moreover, clinical studies involving cocoa interventions are scarce, and they are mainly focused on cocoa's effects on endothelial function and cardiovascular health [42]. Focusing on the exercise field, there are some clinical studies [2,43,44] and some reviews [28,45,46] that assess the ergogenic and antioxidant potential of cocoa and its polyphenols in athletes; however, the results are not clear enough to draw any solid conclusions. In addition, most of the studies assessed the effects of the acute intake of cocoa without focusing on the long-term effects and do not evaluate the impact on immune function. In the current study, we have studied the potential protective effect of a dietary intervention with cocoa on the overproduction of ROS and the consequent immune function impairment induced by intensive running exercise in rats. We have also approached the effect of a dietary intervention with isolated cocoa fiber in these variables, which provides far fewer polyphenols but, in previous studies, has also demonstrated antioxidant and immunomodulating properties [29,31].

Here, both cocoa- and cocoa fiber-enriched diets prevented the increase in ROS production by peritoneal macrophages induced by acute exercise. The preventive effect of the diets may be attributed to the scavenging activity of polyphenols, since due to their chemical structure they can directly neutralize free radicals such as superoxide anions, hydroxyl, peroxynitrite, and nitric oxide radicals by transferring their own electrons [47]. Other mechanisms may be involved, since other polyphenols such as the flavonol quercetin have been shown to stimulate mitochondrial biogenesis throughout enhancing the expression of SIRT1 and PGC-1α, as well as increasing the cytochrome C concentrations in the muscles and brain [22]. Moreover, resveratrol, a non-flavonoid polyphenol, has also shown to reduce oxidative stress through Nrf2 signaling pathway activation [23]. Our results suggest that the intake of a low dose of polyphenols may be enough to prevent an overproduction of ROS, since the CF diet also offers protection against this increase and provides about 10 times fewer polyphenols than the C10 diet. In line with this, other authors have reported a protective effect of a seven-week dietary intervention, with a 5% cocoa fiber-enriched diet (the same dosage as here) on oxidative stress in adipose tissue of obese rats [29]. The protective effect of cocoa on the excessive ROS production induced by exercise agrees with its effect on the oxidative stress induced by adjuvant arthritis, i.e., a model of chronic inflammation [36]. At the clinical level, a study performed in young elite male football players showed that the consumption of 40 g of dark chocolate (providing 799 µg gallic acid equivalents/mL) per day for 30 days was able to prevent oxidative stress, because the diet prevented the increases in serum soluble NADPH oxidase 2-derived peptide levels, the H_2O_2 production, and breakdown activity, as well as the muscle damage observed in control subjects [24]. Other authors also reported the inhibition of the increase in lipid peroxidation induced by exercise after a seven-day cocoa intake [43]. However, they did not observe changes in total plasma antioxidant capacity [43], which agrees with another study showing no changes in the urinary concentration of isoprostanes, which is another

oxidative stress biomarker, in rugby players after a seven-day supplementation with a chocolate beverage [48].

On the other hand, polyphenols also exert their antioxidant effects by inhibiting enzymes such as xanthine oxidase, NADPH oxidase, and tyrosine and protein kinases involved in the production of free radicals [47], as well as by enhancing the endogenous scavenging antioxidant systems [47]. In previous studies, we found higher thymus superoxide dismutase and catalase activities after a dietary intervention with cocoa in healthy rats [49] and a normalization of both enzymatic activities in the spleen of rats with adjuvant arthritis [36]. Moreover, other authors reported an increase in glutathione reductase activity in mice submitted to downhill running after eight weeks of dietary intervention with several catechins [50]. To our knowledge, there are no available studies assessing the effect of cocoa on the activity of these enzymatic systems in the context of the oxidative stress induced by intensive exercise. Therefore, in further studies, we will assess the effect of a cocoa dietary intervention on the endogenous antioxidant systems after intensive exercise, throughout the study of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activities, as well as quantifying the reduced and oxidized glutathione concentrations.

Whereas the endogenous production of ROS is essential for maximal force production and training adaptations, an excessive ROS production can affect muscle force by inducing skeletal muscle fatigue and damage, thus leading to a decrease in performance [51]. Here, the rats just ran to exhaustion once at the end of the study, and then, we could not assess the effect of the increase in ROS production on exercise performance. However, we evaluated the potential ergogenic effect of the experimental diets, including cocoa or cocoa fiber. Neither cocoa- nor cocoa fiber-enriched diets modified the exercise performance of the rats. These results agree with most of the human studies considering cocoa polyphenols [28]. With regard to preclinical studies, the available evidence is quite controversial: some studies reported improvements [7,52,53], and others revealed no changes [8] or even a decrease in exercise performance after dietary interventions with cocoa or its isolated polyphenols [54]. Future studies may clarify how the antioxidant properties of cocoa and cocoa fiber, by means of preventing the overproduction of ROS, affect exercise performance in a chronic manner.

On the other hand, an excessive ROS production can also disrupt immune function and induce an inflammatory status [55], being at least partially responsible for the detrimental effects of intensive exercise on the immune system. A single bout of intensive exercise is able to modify the number and composition of circulating leukocytes [56]. An immediate increase of leukocytes, mainly due to an increase in neutrophils and lymphocytes, followed by a secondary lymphopenia has been extensively described [57]. These alterations are attributed to changes in the hemodynamics, such as vasodilation and increases in heart rate, cardiac output, and blood flow and the action of catecholamines and glucocorticoids on the β2-adrenergic receptors of blood leukocytes in response to exercise [56]. It is well-known that exercise activates the sympathetic nervous system, leading to an increased release of catecholamines and cortisol in a proportional way, i.e., the greater the exercise intensity, the greater the neuroendocrine response [58]. These hormonal changes are normally transient and return to baseline relatively quickly; however, when the intensity of exercise is high and there is not a prior adequate training, the circulating levels of these hormones can be significantly depressed below basal values during the recovery phase [58]. This could explain the decreased plasma levels of cortisol observed in the runner rats 16 h after performing the exhaustion test. These neuroendocrine changes induced by exercise may be responsible for the modifications in the proportions of the circulating granulocytes and lymphocytes. The cocoa diet also decreased the plasma cortisol concentration in the sedentary control group, which agrees with the findings of some clinical studies [59,60]. On the other hand, the impact of acute exercise on the hemoglobin and hematocrit values remains unclear [61]. Most of the available studies, both clinical [61–63] and preclinical [64], reported transient decreases in these values due to plasma volume

expansion, whereas others found no changes in them after exercise [65,66]. Here, probably due to the timing of sampling (16 h after the exercise bout), the protocol applied did not modify the blood erythrocyte counts, the hemoglobin concentration, or the hematocrit. However, the cocoa intake, especially the diet that contained whole cocoa, induced increases in erythrocyte counts, hemoglobin, and hematocrit. These results are in line with the protective effect of cocoa flavanols and procyanidins on the erythrocyte hemolysis induced by excessive free radicals reported both in humans [67] and rats [68], although the observed increases in erythrocyte variables are also present in the non-exercised animals. Further studies may confirm these results and elucidate the potential mechanisms underlying the higher erythrocyte counts found after cocoa consumption. This could be interesting when considering cocoa or any of its components as a potential ergogenic supplement, because it may enhance muscle oxygenation.

Acute intensive exercise also impaired blood Ig concentrations. We found that the acute exercise program applied induced a decrease in serum IgG concentration while those of IgA and IgM were not affected. These findings disagree with those reported previously in a chronic rat model of intensive running exercise [26] and with several clinical studies that reported higher levels of IgG in serum immediately after running 90 km [69], although others did not find differences after five weeks [70] and 14 weeks of running training [71]. These controversial results denote the importance of considering the kind of exercise program performed when comparing results among studies. It seems that a chronic moderate-intensity exercise training increases IgG half-life [72], which could explain the higher serum levels of IgG found by others [26,69–71], as well as inducing beneficial changes in IgG N-glycosylation that may improve its affinity with the Fc receptor [73]. To our knowledge, the impact of a single bout of intensive exercise without a prior training on serum levels of IgG has not been described before. In fact, most of the studies evaluating the impact of exercise on humoral response focus on salivary IgA for their essential role in mucosal immunity. Anyway, the experimental diets with cocoa and cocoa fiber also decreased serum levels of IgG in the sedentary control rats, and there was not an additional decrease by exercise. Moreover, both the C10 and the CF diets lowered the serum content of IgA in runner rats, and the C10 groups also had lower serum levels of IgM, which agrees with the results of previous studies carried out in non-exercised rats and seems to be attributed to the theobromine content of cocoa [4,12].

5. Conclusions

In conclusion, the dietary interventions with cocoa for 25 days were able to avoid the overproduction of ROS induced by a single bout of intensive running exercise, although they were not able to increase the exercise performance or to prevent changes in plasma IgG. Since the cocoa fiber diet also protected against oxidative stress, we can hypothesize that a small content of polyphenols can be enough to counteract this effect. This, together with the beneficial effects of cocoa fiber in the mucosal compartment [11,31,34], such as the prevention of the changes in salivary IgM, the improved cecal short chain fatty acid production, and the decreased secretion of proinflammatory cytokines, makes it a suitable potential supplement to prevent oxidative stress and the microbiota changes induced by an acute intensive exercise. Further research may evaluate the influence of cocoa and cocoa fiber in a more intensive or a longer exercise protocol, which may exacerbate the observed alterations, focusing also on their impact on the endogenous antioxidant systems. It would also be interesting to assess their impact when there is a comorbidity with a disease linked to oxidative stress and their potential protective effect on the induction of an infectious process after exercise, as well as to study possible synergistic combinations with other polyphenols or nutraceuticals.

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ARTICLE 4

"A cocoa diet can partially attenuate the alterations in microbiota and mucosal immunity induced by a single session of intensive exercise in rats"

Patricia Ruiz-Iglesias, Malén Massot-Cladera, Maria J. Rodríguez-Lagunas, Àngels Franch, Mariona Camps-Bossacoma, Margarida Castell, Francisco J. Pérez-Cano.

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- **14ª reunión de la red Española de bacterias lácticas: Bacterias lácticas en alimentación y salud.** Virtual, September 2021. Ruiz-Iglesias P.; Massot-Cladera, M.; Pérez-Cano, FJ.; Castell, M. "**Efecto del cacao y su contenido en fibra sobre la microbiota de ratas sometidas a ejercicio físico agudo**".
- **XII International Society for Immunonutrition Conference.** Virtual, July 2021. Ruiz-Iglesias P.; Massot-Cladera, M.; Azagra-Boronat, I.; Franch-Masferrer, A.; Pérez-Cano, FJ.; Castell, M. "**Cocoa Modulates Mesenteric Lymph Nodes Composition and Function in Rats Submitted to Acute Running Exercise**".
- **XII International Society for Immunonutrition Conference.** Virtual, July 2021. Ruiz-Iglesias P.; Périz, M.; Rio-Aige, K.; Rodríguez-Lagunas, M.J.; Pérez-Cano, FJ.; Castell, M. "**Changes in Peyers' Patches and Intestinal IgA in Acute-Exercised Rats Consuming a Cocoa Diet**".

- **World of Microbiome Digestive and Metabolic Health.** Virtual, November 2020. Ruiz-Iglesias P.; Gómez-Bris, R.; Camps-Bossacoma, M.; Massot-Cladera, M; Franch, À.; Castell, M.; Pérez-Cano, FJ. "**Dietary interventions with cocoa and cocoa fibre affect gut microbiota composition and function in rats submitted to acute physical exercise**".

ABSTRACT

Aim: To establish the influence of cocoa and cocoa fibre on the mucosal immune system and the caecal microbiota composition and function in acutely exercised rats.

Methods: Wistar rats were fed either a standard diet, a diet containing 5% cocoa fibre or a diet containing 10% cocoa. After 4 weeks, half of the rats remained sedentary and the second half run in a treadmill until exhaustion. Sixteen hours later, salivary glands, Peyer's patches, mesenteric lymph nodes and caecal content, among other intestinal samples, were obtained.

Results: The cocoa fibre-enriched diet prevented the exercise-induced increase in faecal humidity, up-regulated the small intestine gene expression of TLR7 and enhanced the caecal concentration of short chain fatty acids, avoiding the reduction in propionic acid induced by acute exercise. The cocoa-enriched diet increased the small intestine weight and the number of bacteria in caecal content, although it reduced the proportion of IgA-coated bacteria and *Clostridium coccoides/Eubacterium rectale*. The cocoa diet also normalized the changes induced by acute exercise in the proportion of Tαβ cell subsets (Th and Tc) and Tγδ cell subsets (Tγδ CD8αα and Tγδ CD8αβ) in MLN and PPs, respectively, and decreased the release of the proinflammatory cytokine IFN-γ by MLN lymphocytes

Conclusion: Both cocoa and cocoa fibre exert beneficial effects on intestinal immunity and may help preventing the alterations induced by acute intensive exercise, nevertheless, the different results obtained after both interventions denote interactions between cocoa fibre and the other bioactive compounds present in cocoa.

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A Cocoa Diet Can Partially Attenuate the Alterations in Microbiota and Mucosal Immunity Induced by a Single Session of Intensive Exercise in Rats

Patricia Ruiz-Iglesias[1,2], Malén Massot-Cladera[1,2], Maria J. Rodríguez-Lagunas[1,2], Àngels Franch[1,2], Mariona Camps-Bossacoma[1,2], Margarida Castell[1,2,3] and Francisco J. Pérez-Cano[1,2]*

[1] Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), Barcelona, Spain, [2] Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), UB, Santa Coloma de Gramenet, Spain, [3] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain

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***Correspondence:**
Margarida Castell
margaridacastell@ub.edu

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Background: Following intensive sports events, a higher rate of upper respiratory tract infections and the appearance of gastrointestinal symptomatology have been reported. We aimed to evaluate the effect of a cocoa-enriched diet on the cecal microbiota and mucosal immune system of rats submitted to high-intensity acute exercise, as well as to elucidate the involvement of cocoa fiber in such effects.

Methods: Wistar rats were fed either a standard diet, a diet containing 10% cocoa providing 5% fiber and a diet containing only 5% cocoa fiber. After 25 days, half of the rats of each diet performed an exhaustion running test. Sixteen hours later, samples were obtained to assess, among others, the cecal microbiota and short chain fatty acids (SCFAs) composition, mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) lymphocyte composition, and immunoglobulin (Ig) content in salivary glands.

Results: The intake of cocoa, partially due to its fiber content, improved the SCFA production, prevented some changes in PPs and in MLNs lymphocyte composition and also decreased the production of proinflammatory cytokines. Cocoa diet, contrary to cocoa fiber, did not prevent the lower salivary IgM induced by exercise.

Conclusion: A cocoa dietary intake can partially attenuate the alterations in microbiota and mucosal immunity induced by a single session of intensive exercise.

Keywords: acute exercise, cocoa fiber, IgA, lymphocytes, polyphenols, SCFA, tight junctions

BACKGROUND

Nutrition is important to meet the body's energy and structural needs, as well as to improve the functionality of various body systems. Among these, immune function can be improved by several foods (1). On the other hand, although everybody is aware of the healthy influence of regular moderate-intensity training, physical exercise is often confined to one-time exercise bouts with no prior adequate training. In fact, participation in running races has become increasingly popular

during recent decades. Following intensive sports events, a higher rate of mucosal infections, such as upper respiratory tract infections (URTIs) (2), and the appearance of gastrointestinal symptomatology have been reported (3, 4). A single bout of intense exercise is able to alter the counts of circulating leukocytes (5), especially T lymphocytes, probably due to both a redistribution within lymphoid and non-lymphoid organs (6) and an increased apoptosis of highly differentiated T cells (7). While the spleen seems to act as the main donor organ of T lymphocytes in the redistribution induced by exercise, Peyer's patches (PPs) are one of the main receiving compartments (6, 8). T lymphocyte function can also be impaired following acute exercise, for instance a decreased proliferation ability (9) and a reduced type 1 T helper (Th1) cell cytokine production have been reported (10). Changes in salivary immunoglobulin (Ig) A, as a marker of mucosal immunity, have been widely studied in the field of exercise immunology (10). Although most of the studies point to a decrease in salivary IgA following intense chronic exercise (11), there is no consensus on the impact of acute exercise on this variable (12). Moreover, acute exercise alters intestinal motility, villi structure (13) and increases the gut permeability (14), probably through the alteration of tight junction (TJ) protein expression, redistribution or phosphorylation state (15). Exercise also modifies the intestinal microbiota composition and function (13, 16), as well as its crosstalk with the host, since, among other changes, a decrease in the cell-surface toll like receptor (TLR) expression has also been described (17).

Cocoa consumption has shown antioxidant (18), anti-inflammatory (19) and immunomodulatory (20) properties. These effects are mostly attributed to cocoa flavonoids, mainly (-)-epicatechin, catechin and procyanidins (21), although cocoa also contains other bioactive compounds such as theobromine and a high proportion of fiber (22). Clinical evidence suggests that cocoa and its flavonoids may be useful for the nutritional management of cardiovascular diseases, obesity and diabetes, among other chronic diseases (23), as well as for the prevention of allergies (24). Moreover, although cocoa does not show a clear ergogenic effect in humans, unlike other polyphenol sources (25), its regular intake can be helpful to avoid the intensive exercise-induced oxidative stress (18). On the other hand, preclinical studies have demonstrated cocoa's ability to modulate both systemic and intestinal immune function (20). In this regard, cocoa intake in rats favors the Th1 response, modulates the mucosal IgA secretion and induces beneficial changes in the composition and function of the gut microbiota, for instance increasing the production of short chain fatty acids (SCFAs) and inducing a differential pattern of TLR gene expression (20, 22, 26). In addition, polymeric cocoa polyphenols are not absorbed in the small intestine and are metabolized in the colon by the gut microbiota, generating secondary bioactive metabolites that are then bioavailable and may exert even greater effects than the original polyphenols (27). Both the cocoa polyphenols and their secondary metabolites can modulate the microbiota composition and function (22, 27). This, together with the high percentage of dietary fiber present in cocoa, can explain the prebiotic properties that have been attributed to cocoa (22, 27).

Considering this background, we hypothesize that cocoa intake can prevent the intestinal and immune alterations induced by a single intense exercise bout. Hence, we aimed to evaluate the effect of a cocoa-enriched diet on the physical performance, the cecal microbiota and the mucosal immune system of rats submitted to high-intensity acute exercise, as well as to elucidate the involvement of cocoa fiber in such effects.

MATERIALS AND METHODS

Animals

Forty-eight female Wistar rats (4-week-old) were obtained from Envigo (Huntingdon, United Kingdom) and maintained in polycarbonate cages (4 rats per cage) in the animal facilities of the Faculty of Pharmacy and Food Science at the University of Barcelona. Female rats were used because previous studies (28–30) showed a better adaptation to the treadmill in female than in male rats, while the immunological variables assessed were not modified by gender (28, 29). Animals were kept under conditions of controlled temperature and humidity in a 12:12 light-dark cycle and had access to food and water *ad libitum*. Animal handling was carried out on a scheduled basis (from 9:00 a.m. to 12:00 p.m.) to avoid the influence of biological rhythms. Rat body weight and the intake of food and water per cage were monitored throughout the study. The animal procedure was approved by both the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEEA/UB ref. 517/18 and DAAM 9257, respectively).

Diets and Acute Exercise Protocol

The acute exercise protocol applied was based on the one reported by Chaves et al. (13), with some adaptions. Firstly, to avoid biased distribution of animals, and before starting experimental diets, all rats were adapted to a specialized treadmill (Exer3/6, Columbus, OH, United States) for 1 week by increasing progressively both the running time and the treadmill speed (starting with speed 0). Then, the animals were subjected to a preselection exhaustion test (ET), which consisted of running 10 min at 18 m/min, and from then on, increasing the speed 3 m/min every 2 min until exhaustion, which was defined as the time that rats remained at the shock grid or they touched it more than three times. After checking the ability to run in the preselection ET, rats were homogeneously distributed into 6 groups (n = 8/each): REF/C, REF/R, C10/C, C10/R, CF/C, and CF/R that will receive three different diets. C (i.e., control) groups refer to the animals that did not run a final bout of ET (see later), and R (i.e., runner) groups refer to those that ran a final bout of ET. The REF groups (i.e., REF/C and REF/R groups) were fed with the standard diet AIN-93M (maintenance diet from the American Institute of Nutrition, Envigo). AIN-93M diet was selected as a reference diet and therefore as a base for the supplemented experimental diets in order to perform the same dietary intervention that was used in previous studies in animals with similar age (26, 31). The C10 groups (i.e., C10/C and C10/R groups) received a special chow with 10% defatted cocoa (Idilia

Foods S.L., Barcelona, Spain). The CF groups (i.e., CF/C and CF/R groups) were fed with a diet containing 5% cocoa fiber powder (Idilia Foods S.L.), which provided a similar fiber cocoa content as the C10 diet but with a lower amount of polyphenols (**Table 1**), as applied in previous studies (32). The experimental chows used in the C10 and CF groups were elaborated on the basis of the AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fiber provided by the cocoa and cocoa fiber powders, resulting being isoenergetic to the REF diet (**Table 1**).

Nutritional intervention lasted for 25 days. In the last week all rats were again adapted to run on the treadmill for 1 week. Afterward, R groups (REF/R, C10/R and CF/R groups) performed a final ET on a treadmill, while C groups (REF/C, C10/C and CF/C groups) did not. The final bout of ET was carried out in the afternoon, between 5:00 and 8:00 p.m., and consisted of running 15 min at 18 m/min, and from then on, increasing the speed 3 m/min every 2 min until exhaustion. The run length was recorded for each rat. The animals were weighted before and after performing the ET to assess the body weight loss. Moreover, all the feces made during the ET were collected and weighted both fresh and after drying, to assess their water content. Simultaneously, feces from the control rats were obtained and weighted in similar conditions with no exercise.

Sample Collection and Processing

Sixteen hours after performing the final ET in order to avoid immediate, transitory effects (in the following day, between 9:00 a.m. and 12:00 p.m.) and without food deprivation, animals were anesthetized intramuscularly with 90 mg/kg of ketamine (Merial Laboratories S.A. Barcelona, Spain) and 10 mg/kg of xylazine (Bayer A.G., Leverkusen, Germany) and exsanguinated. Urine, feces, mesenteric lymph nodes (MLNs), small intestine, cecal content (CC) and submaxillary salivary glands (SMGs) were collected.

The small intestine was weighted and then a 0.5 cm piece (20–30 mg of tissue) of its middle part was immediately immersed in RNAlater® (Ambion, Life Technologies, Madrid, Spain), kept at 4°C overnight and then stored at −20°C until the RNA extraction. The rest of the small intestine was flushed with cold phosphate buffered saline, pH 7.2 (PBS), to remove its content. Then the proximal part of the small intestine was cut into small pieces, weighed, and incubated in shaking with 4 mL of PBS for 10 min at 37°C (55 shakes/min). After centrifugation (538 *g*, 4°C, 10 min), supernatants (gut wash, GW) were collected and stored at −20°C until IgA quantification (33). On the other hand, the distal half of the small intestine was opened lengthwise in order to collect the visible Peyer's patches (PPs).

The lymphocytes from MLNs (MLNLs) and PPs (PPLs) were isolated by mechanically crushing the tissues through a sterile mesh cell strainer (Thermo Fisher Scientific, Barcelona, Spain) (34). PPs were previously incubated in shaking with 1 mM dithiothreitol (DTT) for 5 min at 37°C (55 shakes/min). The number and viability of the obtained lymphocytes were determined by a Countess Automated Cell Counter (Invitrogen, Barcelona, Spain).

SMGs, CC, and feces were homogenized using a tissue homogenizer (Polytron, Kinematica, Lucerne, Switzerland, for SMG; Pellet Pestle Cordless Motor, Kimble, Meiningen, Germany, for CC and feces), as previously detailed (33). After centrifugation (538 *g* for SMGs and 400 *g* for CC and feces, 4°C, 5 min), the supernatants were collected and stored at −20°C until Ig quantification.

Urine Total Phenolic Content Quantification

The Folin-Ciocalteu method was used to determine the total phenolic content in urine, as previously described (34). Absorbance was quantified on a microplate photometer (Sunrise™, Tecan, Männedorf, Switzerland) and data were interpolated using a gallic acid standard curve (0–32 µg/mL). The polyphenol content was then normalized by urine creatinine concentration, which was measured using the Creatinine Urinary Detection Kit (Invitrogen) according to the manufacturer's instructions.

Immunoglobulin A-Coated Bacteria Proportion

The proportion of IgA-coated bacteria (IgA-CB) was evaluated as an indirect immune biomarker of the intestinal immune response against pathogens (35). IgA-CB in CC was determined by flow cytometry as previously described (26), with minor modifications. In the current study, the CC homogenate was stained with rabbit anti-rat Ig polyclonal antibody conjugated to fluorescein isothiocyanate (FITC) (Abcam, Cambridge, United Kingdom). Bacteria were gated in a FacsAria SORP sorter (BD Biosciences, Madrid, Spain), after propidium iodide (PI) staining (Sigma-Aldrich, Madrid, Spain) and according to their forward (FSC) and side scatter (SSC) characteristics. Analysis was performed in the Flow Cytometry Unit (FCU) of the Scientific and Technological Centers of the University of Barcelona (CCiTUB) using the FlowJo v.10 software (Tree Star, Inc., Ashland, Oregon, United States).

Microbiota Analysis by Fluorescence *in situ* Hybridization Coupled to Flow Cytometry

Two of the most studied bacterial groups involved in the microbiota changes induced by exercise, the *Clostridium coccoides/Eubacterium rectale* and *Lactobacillus/Enterococcus* were determined by fluorescence *in situ* hybridization coupled to flow cytometry (FISH-FCM) analysis using group-specific fluorochrome-conjugated probes (Erec482 5′-GCTTCTTAGTCARGTACCG and Lab158 5′-GGTATTAGCAYCTGTTTCCA, respectively) (Sigma-Aldrich), which hybridize the bacterial 16S RNA of each particular group as previously detailed (26). Data were acquired by a FacsAria SORP sorter (BD Biosciences) in the FCU of the CCiTUB and the analysis was performed with FlowJo v.10 software (Tree Star). The results were normalized by total bacteria, which was detected adding PI (Sigma-Aldrich) prior to the flow cytometry acquisition. Commercial Flow Check™

Fluorospheres (Beckman Coulter, Inc., FL, United States) were used to assess the total counts of bacteria.

Short Chain Fatty Acid Analysis

Acetic, propionic, butyric, valeric, and isobutyric acids were quantified in CC homogenates by headspace gas chromatography-mass spectrometry (HSGC-MS) at the GC-MS Unit of the CCiTUB, as previously detailed [31]. A 10 mM volatile free acid mix serial dilution (Supelco, Bellefonte, PA, United States) containing acetic, propionic, butyric, valeric and isobutyric acids was used as standard. The lower limits of detection were 0.404 μmol/g for acetic acid, 0.068 μmol/g for propionic acid, 0.020 μmol/g for butyric acid, 0.001 μmol/g for valeric acid, and 0.003 μmol/g for isobutyric acid.

Immunoglobulin Quantification

The concentration of IgA and/or IgM in SMG, GW, CC, and feces were determined by a sandwich ELISA (Bethyl Laboratories Inc., Montgomery, TX, United States), as previously explained (36). Absorbance was quantified on a microplate photometer (Labsystem Multiskan, Helsinki, Finland) and data were interpolated by Ascent v.2.6 software (Thermo Fisher Scientific) according to the respective standard curves. The Ig content in SMG, CC and feces was normalized by the total protein concentration, which was determined using the Pierce-660 nm ready-to-use Protein Assay Reagent (Thermo Fisher Scientific) following the manufacturer's instructions.

Peyer's Patches and Mesenteric Lymph Nodes Lymphocyte Phenotypic Analysis

MLNLs and PPLs were extracellularly stained with mouse anti-rat monoclonal antibodies (mAb) conjugated to either FITC, phycoerythrin (PE), peridinin-chlorophylla protein (PerCP), allophycocyanin (APC) and brilliant-violet 421 (BV421), as previously detailed (37). The fluorochrome-conjugated mAb used were targeted to TCRαβ, CD4, CD8α, CD8β, NKR-P1A (CD161), TCRγδ, and CD45RA (BD Biosciences). A negative control staining without any mAb and a staining control for each mAb were included. Data were acquired by Gallios Cytometer (Beckman Coulter, Miami, FL, United States) in the FCU of the CCiTUB and the analysis was performed with FlowJo v.10 software (Tree Star). Results are expressed as the proportion of positive cells in the lymphocyte population selected according to their FSC/SSC characteristics or the positive cells in a particular lymphocyte subset.

TABLE 1 | Composition of the experimental diets (g/kg diet).

Components	Reference diet (g/kg)	10% Cocoa diet (g/kg)	5% Cocoa fiber diet (g/kg)
Proteins	141.8	142.6	141.1
Carbohydrates	720.7	697.9	705.1
Lipids	40.0	40.1	38.0
Insoluble fiber	50.0	54.0	56.0
Soluble fiber	0	6.0	8.0
Polyphenols	0	3.6	0.4
Theobromine	0	1.8	0.6
Other (minerals, vitamins,...)	47.5	54.0	50.9

Mesenteric Lymph Nodes Lymphocyte Stimulation and Proliferation

MLNLs (10^5 cells/well) were incubated in 96-well plates (TPP, Sigma-Aldrich) and stimulated or not with concanavalin A (ConA, 5 μg/mL, Sigma-Aldrich). After 48 h, supernatants were collected and stored at −80°C until cytokine production analysis while the MLNLs proliferation capacity was quantified by BrdU Cell Proliferation Assay (Roche, Madrid, Spain). The proliferation rate was obtained by dividing the absorbance of ConA-stimulated cells with that of non-stimulated cells. Results are represented as fold change in the proliferation rate, considering the mean of REF/C group values as 1.

Cytokine Quantification

Interferon (IFN)-γ, interleukin (IL)-2, IL-10, IL-4, IL-6, and tumor necrosis factor (TNF)-α production was determined in ConA-stimulated MLNLs supernatants by ProcartaPlex Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, United States), according to the manufacturer's protocol. Data were acquired by MAGPIX Cytometer (Affymetrix) in the FCU of the CCiT-UB and analyzed by ProcartaPlex Analyst v1.0 software (Affymetrix). The lowest limits of detection were 3.34 pg/mL for IFN-γ, 1.82 pg/mL for IL-2, 6.01 pg/mL for IL-10, 0.62 pg/mL for IL-4, 2.19 pg/mL for IL-6, and 2.88 pg/mL for TNF-α.

Quantification of Gene Expression in Small Intestine

Polymeric Ig receptor (pIgR), retinoic acid receptor (RAR)-α, forkhead box P3 (FoxP3), TLR2, TLR4, TLR5, TLR7, free fatty acid receptor (FFAR) 2, FFAR3, occludin (Ocldn), claudin-4 (Cldn4), zonula occludens (ZO)1, and ZO2 gene expression were assessed in the small intestine, as previously described (37). Briefly, the small intestine portion kept in RNA later was homogenized in a lysing matrix tube (MP Biomedicals, Illkirch, France) by a FastPrep-24 instrument (MP Biomedicals) for 30 s and the RNA contained was isolated with the RNeasy mini kit (Qiagen, Madrid, Spain). RNA was quantified using a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, United States) and reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany). Afterward, the Real Time (RT)-PCR was carried out using the ABI Prism 7900 HT quantitative RT-PCR system (AB) and the following specific PCR TaqMan primers (AB): pIgR (Rn00562362_m1, Inventoried, I), RARα (Rn00580551_m1, I), FoxP3 (Rn01525092_m1, I), TLR2 (Rn02133647_s1, I), TLR4 (Rn00569848_m1, I), TLR5 (Rn04219239_s1, I), TLR7 (Rn01771083_m1, I), FFAR2 (Rn02345824_s1, I), FFAR3 (Rn01457614_g1, I), Ocldn (Rn00580064_m1, I), Cldn4 (Rn01196224_s1, I), ZO1 (Rn02116071_s1, I) and ZO2 (Rn01501483_m1, I). Data was analyzed with SDS version 2.4 software (AB). The housekeeping genes Gusb (β-glucuronidase, Rn00566655_m1, I) and β-actin (Rn00667869_m1, I) were used for normalizing the gene expression of the target genes, using

the $2^{-\Delta\Delta Ct}$ method (38). Since the target and the housekeeping genes should have a similar expression level, the gene expression of β-actin was used as housekeeping gene for pIgR, RARα, TLR5, Ocldn, Cldn4, and ZO2, whereas Gusb expression was used for the rest of the genes. Fold changes in small intestine gene expression are expressed considering the mean of REF-C group values as 1.

Statistical Analysis

Statistical analysis of the data was performed using IBM Social Sciences Software Program (SPSS, version 26.0, Chicago, IL, United States). Normality and variance equality of the data were confirmed by Shapiro-Wilk's and Levene's test, respectively. A two-way ANOVA test was applied and, if significant differences were detected, Tukey's *post-hoc* test was carried out. When results were neither equally nor normally distributed, a Kruskal-Wallis test was used and, if significant differences were detected, Dunnet's test was performed as a *post-hoc* test. When comparing variables throughout the study (e.g., body weight, chow intake and water intake), the repeated measures ANOVA test was used to assess whether there was a significant interaction between time (day of study) and the dietary condition (REF, CF and C10 diets). Once we confirmed there was a significant interaction, a one-way ANOVA test followed by the Tuckey's *post-hoc* test was used to detect between which days of study the differences between the dietary groups were statistically significant. Significant differences were considered when $p < 0.05$.

RESULTS

Body Weight, Chow Intake, Urine Flavonoid Content, and Exercise Performance

Body weight (BW), chow and water intake were monitored every 3–4 days throughout the 25-days nutritional intervention (**Figure 1**). The BW increased progressively during the study period in all dietary groups (time effect $p = 0.001$ by repeated-measures ANOVA), but the animals that received the C10 diet had a lower BW gain than the REF and CF groups (**Figure 1A**) (Time x Diet interaction effect $p = 0.001$ by repeated-measures ANOVA), while they consumed a similar amount of chow than REF animals (**Figure 1B**). The water intake was much higher in the C10 group during the first week of nutritional intervention (**Figure 1C**).

To confirm the intestinal absorption of the polyphenols contained in cocoa diets, the total polyphenol content in urine was quantified at the end of the study in the six groups of animals (**Figure 1D**). The C10 diet (both C10/C and C10/R groups), with the highest content of polyphenols, significantly increased the total urine polyphenol content.

During the final ET, the distance run, the total fecal mass produced, and the BW loss were monitored (**Figure 2**). The REF animals ran more than 900 m, produced about 1 g of feces and lost about 1.7 g of BW during the final ET. Neither C10 nor CF diets significantly modified these variables. However, the CF enriched diet tended to decrease the total fecal mass and the BW loss compared to the other runner groups ($p < 0.1$).

Fecal Water Content, Cecal pH, and Small Intestine Weight

The influence of a single bout of intensive exercise and the cocoa-enriched diets on fecal humidity (water content), cecal pH and small intestine weight were also assessed at the end of the study (**Table 1**). Feces during intensive exercise had a higher water content but the CF diet—but not C10 diet—avoided this increase. There were no changes in the cecal pH (**Table 2**).

Exercise did not modify the small intestine weight, independently of the dietary condition (**Table 2**). However, following the C10 diet, the small intestine weight rose.

Cecal Microbiota Composition

The amount of cecal bacteria, the proportion of them coated to IgA (IgA-CB), as well as the cecal proportion of *Clostridium coccoides/Eubacterium rectale* and *Lactobacillus/Enterococcus* in CC were established in samples obtained 16 h after the final exhaustion (**Figure 3**). Acute exercise did not modify these variables. However, the consumption of the C10 diet, but not that of CF, increased, by more than threefold, the amount of total bacteria in the cecum in both exercised conditions ($p < 0.01$). Moreover, C10 diet resulted in a lower proportion of IgA-CB in runner rats ($p < 0.05$). C10 consumption, but not that of CF, decreased the proportion of *C. coccoides/E. rectale* in both exercise conditions ($p < 0.01$) without modifying that of *Lactobacillus/Enterococcus.*

Cecal Short Chain Fatty Acids Production

The function of the gut microbiota was assessed by means of cecal SCFA quantification (**Figure 4**). Overall, acute exercise significantly decreased the production of propionic ($p < 0.05$) and tended to lower that of valeric acid ($p < 0.1$). On the other hand, runner CF rats, but not C10 rats, showed a higher total SCFA concentration in cecum content than REF rats, which was mainly due to an increase in acetic acid production, the most abundant SCFA. Both CF and C10 diets prevented the decrease in propionic acid induced by exercise, although these diets did not avoid the changes in valeric acid concentration. Neither acute exercise nor the experimental diets modified the production of butyric acid. In addition, CF and C10 diets decreased the amount of isobutyric acid in sedentary control and runner rats ($p < 0.05$).

Mucosal Immunoglobulins

Mucosal Ig concentrations were determined in SMG, GW, CC, and feces, 16 h after the final ET (**Figure 5**). In SMG, acute exercise induced a 30% decrease in IgM content ($p = 0.01$) and tended to decrease that of IgA (**Figures 5A,B**). CF diet intake prevented the decrease in IgM levels. The C10 diet decreased the IgM and IgA content in SMG in both exercise conditions.

Regarding the intestinal IgA content, the statistical analysis showed an overall decrease in GW IgA levels due to acute exercise, although these changes were only observed in CF and

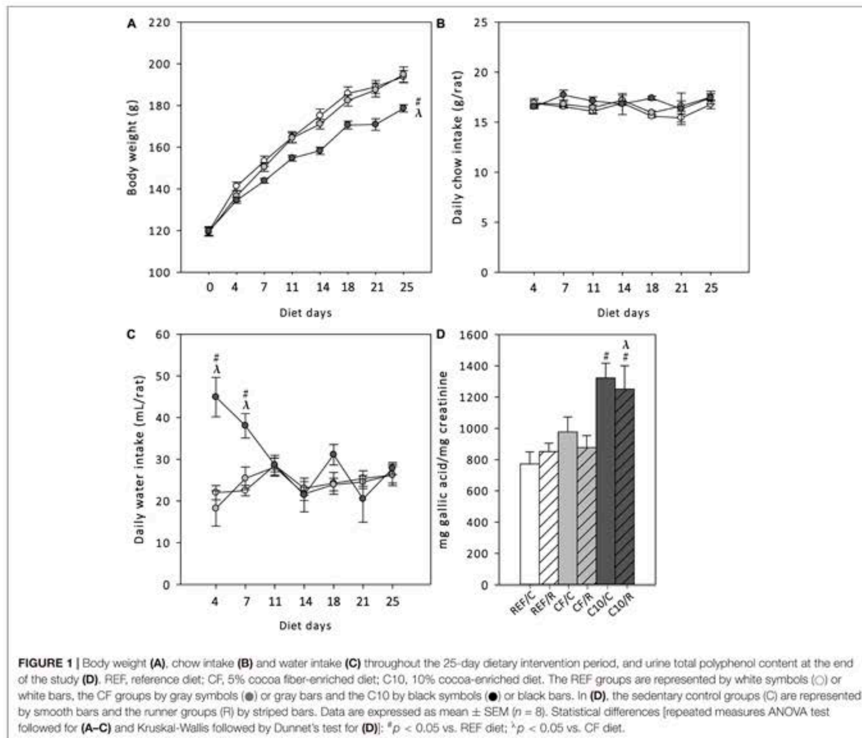

FIGURE 1 | Body weight **(A)**, chow intake **(B)** and water intake **(C)** throughout the 25-day dietary intervention period, and urine total polyphenol content at the end of the study **(D)**. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. The REF groups are represented by white symbols (○) or white bars, the CF groups by gray symbols (●) or gray bars and the C10 by black symbols (●) or black bars. In **(D)**, the sedentary control groups (C) are represented by smooth bars and the runner groups (R) by striped bars. Data are expressed as mean ± SEM ($n = 8$). Statistical differences [repeated measures ANOVA test followed for **(A–C)** and Kruskal-Wallis followed by Dunnet's test for **(D)**]: $^{\#}p < 0.05$ vs. REF diet; $^{\lambda}p < 0.05$ vs. CF diet.

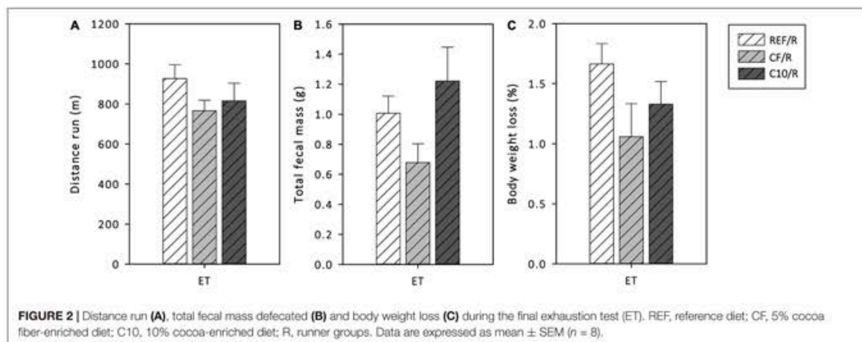

FIGURE 2 | Distance run **(A)**, total fecal mass defecated **(B)** and body weight loss **(C)** during the final exhaustion test (ET). REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet; R, runner groups. Data are expressed as mean ± SEM ($n = 8$).

C10 runner groups (**Figure 5C**). Neither exercise nor the diets significantly modified cecal and fecal IgA (**Figures 5D,E**).

Peyer's Patches Lymphocyte Composition

To assess the influence of a single bout of exercise and the experimental diets on the gut-associated lymphoid tissue, the proportion of the main lymphocyte subsets in PPs was also established 16 h after performing the final ET (**Figure 6**). The proportion of B cells, Tαβ cells and NK cells was not affected by acute exercise or diet. However, the C10 diet, but not the CF diet, increased the proportion of Tγδ cells in sedentary control animals ($p < 0.05$ vs. REF/C and CF/C groups). Concerning the Tαβ cell subsets, there were no changes due to acute exercise but the C10 diet decreased the Th cell proportion and reciprocally increased that of Tc cells in runner animals ($p < 0.05$). Moreover, the C10 diet, but not the CF diet, increased the proportion of NKT cells in sedentary control animals ($p < 0.05$). Regarding the Tγδ CD8αα and CD8αβ cell subsets, runner rats from the REF group showed a lower proportion of Tγδ CD8αα cells and, reciprocally, a higher one of Tγδ CD8αβ cells than sedentary counterparts ($p < 0.05$, REF/C group vs. REF/R group). In this case, the CF diet, but not the C10 diet, induced the same changes as acute exercise ($p < 0.05$, REF/C group vs. CF/C group).

Mesenteric Lymph Nodes Lymphocyte Composition

The phenotypic composition of MLNLs was also characterized 16 h after performing the final ET (**Figure 7**). The acute exercise did not significantly modify the proportion of B cells, Tαβ cells, Tγδ cells, and NK cells (**Figure 7A**). However, the C10 diet increased the proportion of Tγδ cells in the sedentary animals ($p < 0.05$).

Regarding the main Tαβ cell subsets, acute exercise increased the proportions of Th cells and NKT cells ($p < 0.05$ in both cases), with a reciprocal decrease in Tc cell percentage ($p < 0.05$ REF/R vs. REF/C) (**Figure 7B**). The animals fed with the C10 diet showed a lower proportion of Th cells and a higher one of Tc cells than REF and CF rats ($p < 0.05$), and the changes induced by acute exercise were also observed in this dietary group. On the other hand, the CF diet intake prevented the exercise-induced changes in Th and Tc cell proportions while both cocoa-enriched diets attenuated the change in NKT cell proportion. With regard to the Tγδ CD8αα and CD8αβ cell subsets, while exercise did not induce any change, the C10 diet, but not the CF diet, increased the proportion of the Tγδ CD8αα cells ($p < 0.05$ C10/C vs. REF/C and CF/C) while it decreased reciprocally that of Tγδ CD8αβ cells ($p < 0.05$ C10/C vs. REF/C and CF/C) (**Figure 7C**).

TABLE 2 | Water content of feces defecated during the exhaustion test and the same time in the control rats, cecal content pH, and small intestine weight at the end of the study.

	Group	REF	CF	C10
Fecal water content (%)	C	46.12 ± 0.94	48.91 ± 1.21	46.68 ± 0.12
	R	53.68 ± 3.07*	49.96 ± 1.72	53.13 ± 2.34*
Cecal content pH	C	7.63 ± 0.09	7.65 ± 0.11	7.57 ± 0.11
	R	7.65 ± 0.09	7.54 ± 0.12	7.78 ± 0.10
Small intestine weight (g/100 g BW)	C	100.00 ± 2.17	88.46 ± 3.21#	108.66 ± 2.60#λ
	R	94.40 ± 2.80	92.55 ± 2.09#	110.29 ± 2.46#λ

*REF, reference diet; CF, 5% cocoa fiber enriched diet; C10, 10% cocoa enriched diet. Data are expressed as mean ± SEM (n = 8). C, control group; R, runner group. Statistical differences (Kruskal-Wallis followed by Dunnet's test): *p < 0.05 vs. the C group in the same diet; #p < 0.05 vs. the same exercise condition in the REF diet; λp < 0.05 vs. the same exercise condition in the CF diet.*

Mesenteric Lymph Node Lymphocyte Functionality

The functionality of MLN T lymphocytes was assessed by means of their proliferative response and cytokine production ability after ConA stimulation (**Figure 8**). Neither exercise nor the experimental diets affected significantly the MLNLs proliferation capacity (**Figure 8A**).

Acute exercise increased the production of TNF-α in all dietary conditions (**Figure 8G**), without significantly modifying that of IL-2, IFN-γ, IL-10, IL-4, and IL-6. The C10 diet, but not the CF diet, decreased the production of the proinflammatory cytokine IFN-γ in both exercise conditions. On the other hand, the CF diet increased the TNF-α production.

Gene Expression in Small Intestine

The effect of acute exercise and the cocoa diets on the gene expression of some molecules involved in the crosstalk between the microbiota and the host, IgA production and TJ formation were also assessed in small intestine tissue (**Figure 9**).

A single bout of intensive exercise did not modify the gene expression of the studied TLRs (**Figures 9A–D**). CF runner rats, but not those fed with C10, showed a higher expression of TLR7 ($p < 0.05$, vs. CF/C and REF/R). On the other hand, C10 animals, but not CF rats, had lower levels of FFAR3 in both exercise conditions ($p < 0.05$ vs. REF and CF diets).

Regarding the small intestine gene expression of proteins involved in IgA transcytosis and IgA^+ B cell differentiation, acute exercise in REF animals and the intake of the C10 diet increased the pIgR gene expression ($p < 0.05$ in both cases), whereas the combination of both (C10/R) unexpectedly decreased it ($p < 0.05$ vs. REF/R and C10/C) (**Figure 9G**). The expression of RARα and FoxP3 was not affected by either exercise or the experimental diets (**Figures 9H,I**).

On the other hand, a single bout of intensive exercise did not modify the gene expression of the TJ proteins Ocldn, Cldn4, ZO1 and ZO2 in REF animals (**Figures 9J–M**). However, both experimental diets increased by more than twofold the expression of ZO1 ($p < 0.05$ vs. REF diet) in both exercise conditions. In addition, both sedentary and runner C10 animals showed lower levels of ZO2 ($p < 0.05$), whereas the gene expression of Ocldn ($p < 0.05$, REF/R vs. C10/R) and Cldn4 ($p < 0.05$, C10/R vs. REF/R and CF/R) decreased only in C10 runner animals ($p < 0.05$ for Ocldn and Cldn4, C10/C vs. C10/R). These changes were not found in rats submitted to the CF diet.

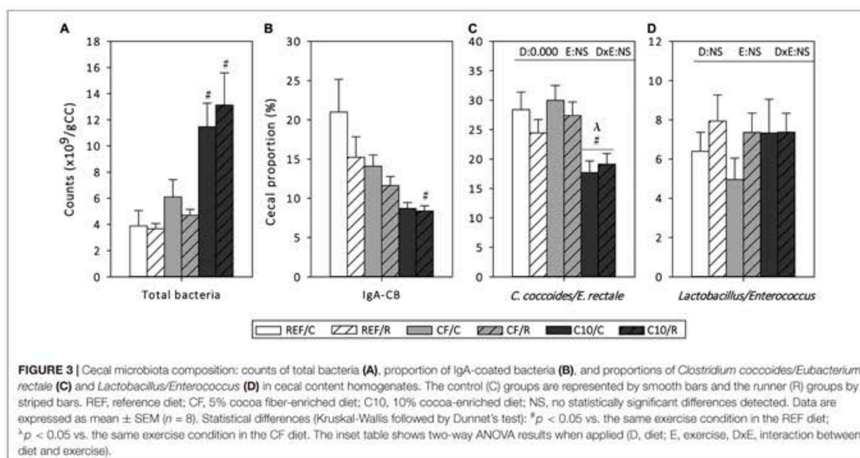

FIGURE 3 | Cecal microbiota composition: counts of total bacteria **(A)**, proportion of IgA-coated bacteria **(B)**, and proportions of *Clostridium coccoides/Eubacterium rectale* **(C)** and *Lactobacillus/Enterococcus* **(D)** in cecal content homogenates. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet; NS, no statistically significant differences detected. Data are expressed as mean ± SEM (n = 8). Statistical differences (Kruskal-Wallis followed by Dunnet's test): $^{\#}p < 0.05$ vs. the same exercise condition in the REF diet; $^{\lambda}p < 0.05$ vs. the same exercise condition in the CF diet. The inset table shows two-way ANOVA results when applied (D, diet; E, exercise, DxE, interaction between diet and exercise).

DISCUSSION

In previous studies, we have demonstrated the immunomodulating (32) and antioxidant properties (31, 39) of cocoa enriched-diets, as well as the prebiotic effects (22) of cocoa fiber in both healthy rats (22, 32) and experimental models of allergy and inflammation (31, 34, 39, 40). However, there are no studies focused on these cocoa properties in a situation of excessive oxidative stress, such as in an acute intensive exercise. In the current study, we aimed to evaluate the influence of a single intense exercise bout and a 25-day cocoa-enriched diet in rats on their cecal microbiota and mucosal immune system. Likewise, we aimed to elucidate the involvement of cocoa fiber in such effects. We found that a single intense exercise bout increased the fecal humidity and, although no change in the cecal microbiota composition was observed, a reduction in some SCFAs, such as propionic acid and a trend to decrease the proportion of valeric acid was found. Moreover, a single bout of intense exercise decreased the IgM content in salivary glands and the proportion of Tγδ CD8αα cells in PPs, whereas increased the percentage of Th lymphocytes in MLNs, and raised the gene expression of the pIgR in the intestinal wall. The cocoa-enriched diet (C10), which did not modify the exercise performance or prevent the exercise-induced increased fecal humidity, was able to increase the small intestine weight and the cecal bacterial content, although it induced a lower proportion of IgA-coated bacteria and *Clostridium coccoides/Eubacterium rectale*, changes that could be due to other bioactive compounds rather than fiber because the CF diet, which contained the same amount of cocoa fiber and no polyphenols or methylxanthines, did not induce these changes. However, cocoa diet, due to its fiber content, avoided the decrease in cecal propionic acid but not that in valeric acid induced by exercise. Moreover, diets containing cocoa or cocoa fiber alone reduced the production of isobutyric acid. In the mucosal Igs, the cocoa diet decreased the salivary IgM and IgA contents. These changes could be explained again by the effect of other cocoa bioactive compounds rather than fiber, since the CF diet did not produce the same effect. In PPs, 25-day cocoa diet caused some changes in lymphocyte composition, partially due to its fiber content, and prevented the decrease in the proportion of Tγδ CD8αα cells, probably due to other bioactive compounds, induced by exercise. Likewise, the cocoa diet, partially because of its fiber content, reduced the proportion of Th cells but increased that of Tγδ and Tγδ CD8αα cells in MLNs, and prevented the increase in NKT cell percentage induced by exercise in this compartment. With regard to the functionality of MLNLs, reduced the production of the proinflammatory IFN-γ. Finally, after 25 days, cocoa diet, but not cocoa fiber, decreased the FFAR3 and ZO2 gene expression, whereas both diets enhanced two-to threefold that of ZO1. With regard to cocoa fiber, on its own it prevented the increase in fecal humidity, raised the production of SCFAs and the TLR7 gene expression, and avoided the decrease in salivary IgM induced by exercise.

Focusing on exercise performance, the cocoa diet did not improve it, which agrees with most of the human studies using cocoa polyphenols (25). Even so, some preclinical studies have reported that (–)-epicatechin and (–)-epicatechingallate, both flavanols present in cocoa, exert ergogenic effects in rodents (41–43), while others reported no changes (44) or even a decrease in time-to-exhaustion after a 6-month dietary intervention (45). The controversy in these results may be due to the dosage of cocoa or its polyphenol content, as well as the exercise protocol applied.

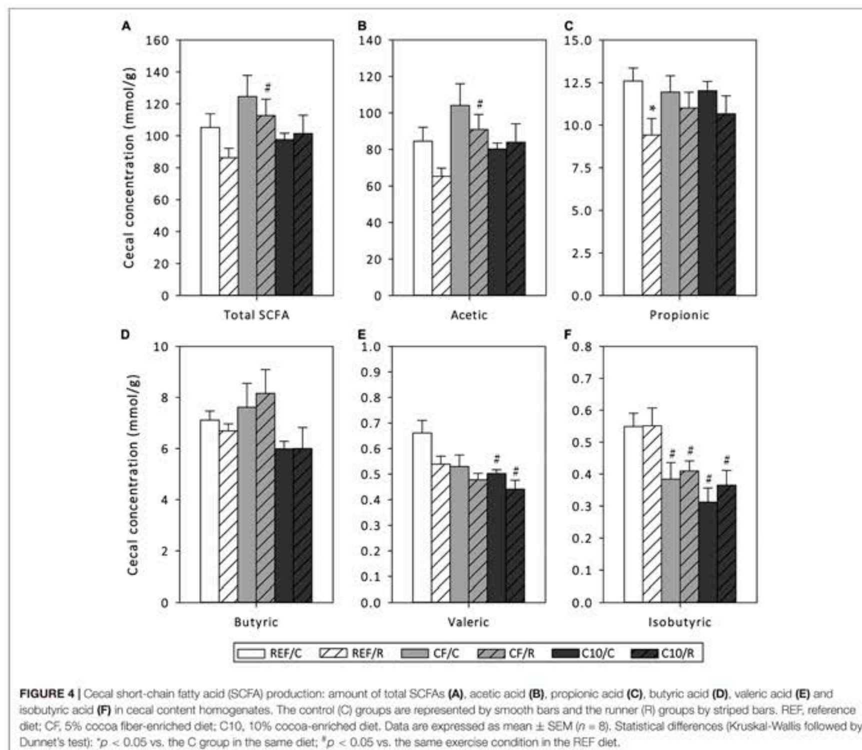

FIGURE 4 | Cecal short-chain fatty acid (SCFA) production: amount of total SCFAs **(A)**, acetic acid **(B)**, propionic acid **(C)**, butyric acid **(D)**, valeric acid **(E)** and isobutyric acid **(F)** in cecal content homogenates. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. Data are expressed as mean ± SEM ($n = 8$). Statistical differences (Kruskal-Wallis followed by Dunnet's test): $^{*}p < 0.05$ vs. the C group in the same diet; $^{\#}p < 0.05$ vs. the same exercise condition in the REF diet.

During the exhaustion test, we measured the BW loss to record the level of dehydration, as well as the total fecal mass produced and its water content, which allowed us to approach changes in gastrointestinal (GI) motility. In line with current literature (4, 46), we found that acute exercise increased fecal humidity which may be due to a faster GI motility. This fact was prevented by the intake of the cocoa fiber diet. The current consensus is to avoid a high intake of fiber 24–72 h before and during intensive exercise to prevent a consequent diarrhea (47), nevertheless, the outcome may depend on the kind of fiber, its source and the ratio of soluble and insoluble fiber consumed. Although a more specific method to evaluate changes in GI motility may be applied in future research, our results suggest that the proportion of cocoa fiber tested exerts protective effects against exercise-induced increased GI motility (48, 49). In addition, the involvement of cocoa components in the crosstalk between neurons, immune cells, and microbes, also known as the intestinal neuro-immune axis (50) also deserves to be studied in depth to ascertain the precise mechanisms involved.

In line with this and with regard to the cecal microbiota composition, acute exercise did not induce changes in the amount of total bacteria, the proportion of those bound to IgA, *Clostridium coccoides/Eubacterium rectale* and *Lactobacillus/Enterococcus* percentages or the cecal pH. Surprisingly, these results disagree with the acute exercise-induced decrease in *Lactobacillus* proportion and the increase of that of *Clostridium* found by Chaves et al. using a similar experimental design (13). Nevertheless, the available evidence is quite controversial, since other authors reported a higher *Lactobacillus* proportion in both intensively and moderately exercised rats (51, 52). Further studies may clarify the relationship established between the degree of the exercise's intensity and its impact on gut microbiota composition in a more exhausting study. On the other hand, the intake of the cocoa-enriched diet

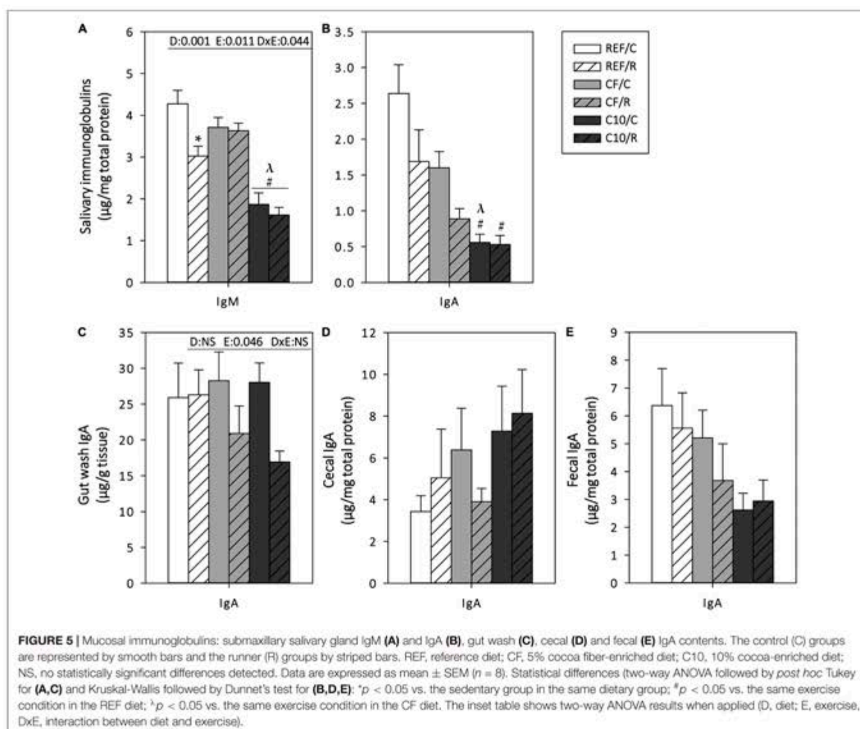

FIGURE 5 | Mucosal immunoglobulins: submaxillary salivary gland IgM **(A)** and IgA **(B)**, gut wash **(C)**, cecal **(D)** and fecal **(E)** IgA contents. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet; NS, no statistically significant differences detected. Data are expressed as mean ± SEM ($n = 8$). Statistical differences (two-way ANOVA followed by *post hoc* Tukey for **(A,C)** and Kruskal-Wallis followed by Dunnet's test for **(B,D,E)**: $^{*}p < 0.05$ vs. the sedentary group in the same dietary group; $^{\#}p < 0.05$ vs. the same exercise condition in the REF diet; $^{\lambda}p < 0.05$ vs. the same exercise condition in the CF diet. The inset table shows two-way ANOVA results when applied (D, diet; E, exercise, DxE, interaction between diet and exercise).

tripled the amount of total bacteria present in cecal content, which could contribute to the higher intestinal weight found in these animals. Moreover, cocoa intake lowered the proportion of bacteria bound to IgA as well as the percentage of *Clostridium coccoides/Eubacterium rectale,* without modifying that of *Lactobacillus/Enterococcus,* results that are in line with previous studies (26). This influence of cocoa in cecal content was not due to its fiber content because the fiber enriched diet did not produce the same changes. Therefore, these changes may be attributed to the other bioactive compounds present in cocoa, such as polyphenols and methylxanthines. In line with this, we have previously reported a similar increase in the amount of total bacteria and a decrease in the proportion of them bound to IgA due to a 4-week hesperidin supplementation, which is one of the main polyphenols found in citrus fruits (53). Theobromine, the predominant xanthine found in cocoa, also seems to play an important role in lowering the proportion of IgA-coated bacteria (54). The impact of these changes depends on the kind of bacteria that was bound to IgA, since IgA can bind pathogenic bacteria to neutralize them or it can transport commensal bacteria to the intestinal epithelium. Nevertheless, it seems that IgA has a higher affinity to inflammatory bacteria (35, 55), since increased levels of this have been found in inflammatory bowel disease patients (35, 56). However, further studies may elucidate the involvement of cocoa's polyphenols and other bioactive compounds in cocoa's impact on cecal microbiota composition.

When assessing the impact on microbiota, it is important to take into account not only the composition but also its function. SCFAs are the main bacterial metabolites of the fermentation of dietary fiber and proteins and they exert an important role as metabolic regulators. Previous research has associated an increased gut-derived SCFA production with a better insulin sensitivity, a reduced inflammation (57) and even an improved exercise performance (58, 59), this last factor being associated with its interaction with skeletal muscle. It has been reported that the regular practice of moderate intensity exercise increases

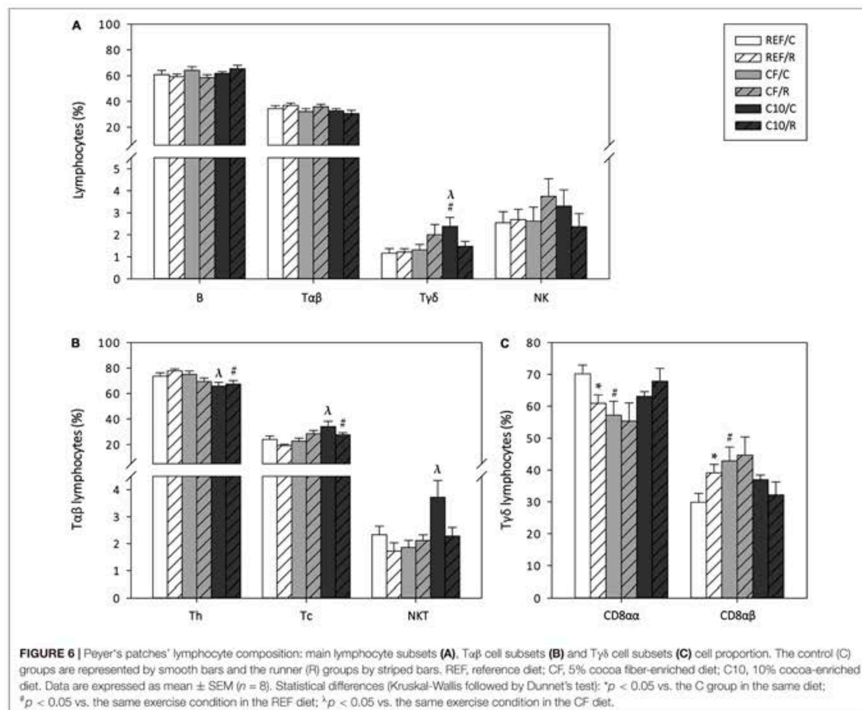

FIGURE 6 | Peyer's patches' lymphocyte composition: main lymphocyte subsets **(A)**, Tαβ cell subsets **(B)** and Tγδ cell subsets **(C)** cell proportion. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. Data are expressed as mean ± SEM (n = 8). Statistical differences (Kruskal-Wallis followed by Dunnet's test): $^{*}p < 0.05$ vs. the C group in the same diet; $^{\#}p < 0.05$ vs. the same exercise condition in the REF diet; $^{\lambda}p < 0.05$ vs. the same exercise condition in the CF diet.

the production of butyric acid (60, 61). Moreover, some authors found an inverse correlation between the fecal concentration of propionic and acetic acids and the exercise performance in a cardiorespiratory fitness test (measured as peak oxygen uptake, VO_2 peak) (60), whereas other studies reported higher propionic and acetic acid levels in elite professionals rugby players (61) and in lean subjects submitted to a 6-weeks endurance-based exercise intervention (62). In spite of these studies focused on long-term exercise, the impact of acute intensive exercise on SCFA production remains unclear. Our present results indicate a lower propionic acid concentration in cecum due to acute exercise, which partially agree with the findings of Estaki et al. (60). Both cocoa- and cocoa fiber- enriched diets attenuated this exercise-induced decrease in propionic acid, and even the CF groups also had higher levels of cecal acetic acid. This agrees with previous studies reporting that a 3-week diet with cocoa fiber increased the cecal concentration of acetic, propionic and butyric acids (22). A recent preclinical study pointed out the importance of acetic acid as an energy substrate during endurance exercise, since a continuous acetic acid infusion was able to restore the lost exercise capacity of mice treated with antibiotics (63). However, we did not observe a higher exercise performance linked to fiber diet. On the other hand, we also found that exercise tended to decrease the cecal content of valeric acid, which together with the branched SCFA isovaleric and isobutyric acids are considered putrefactive SCFAs (pSCFAs). These pSCFAs are products of the bacterial fermentation of proteins and are associated with the generation of other concomitant products that may be detrimental to the colonic epithelium (64). Then, the exercise-induced decrease in valeric acid might be beneficial for the host. Moreover, cocoa-enriched diet, partially because of its fiber content, induced a decrease in the concentration of valeric and isobutyric acids. These results are in line with previous studies that reported a negative correlation between dietary fiber consumption and the levels of pSCFAs (65). Some of the beneficial effects of SCFAs to the host, such as their anti-inflammatory properties, occur through the interaction with the two specific SCFAs receptors FFAR2 (GPR43) and FFAR3

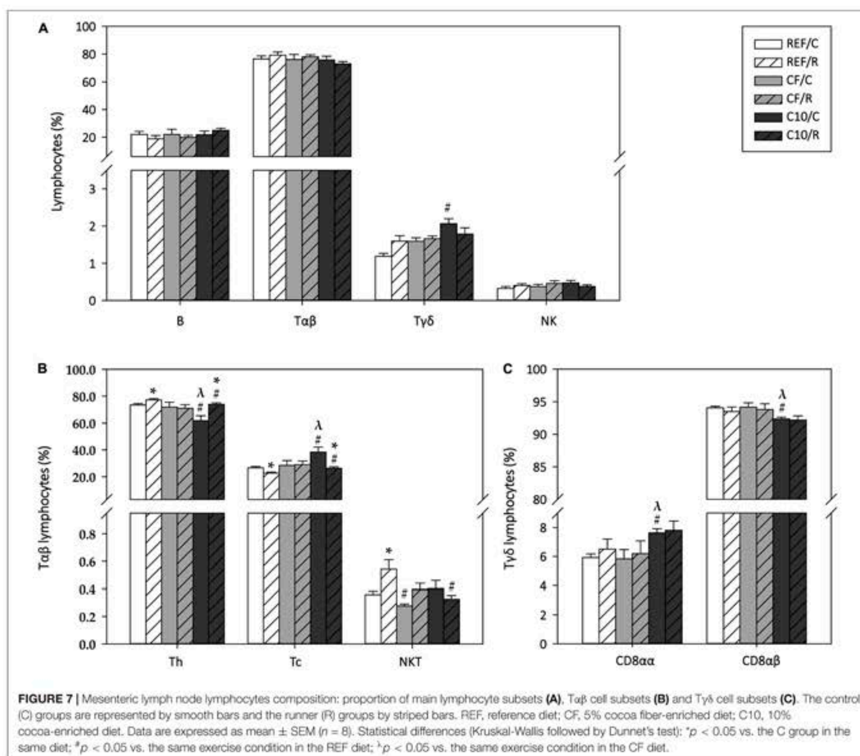

FIGURE 7 | Mesenteric lymph node lymphocytes composition: proportion of main lymphocyte subsets **(A)**, Tαβ cell subsets **(B)** and Tγδ cell subsets **(C)**. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. Data are expressed as mean ± SEM (n = 8). Statistical differences (Kruskal-Wallis followed by Dunnet's test): *$p < 0.05$ vs. the C group in the same diet; #$p < 0.05$ vs. the same exercise condition in the REF diet; λ$p < 0.05$ vs. the same exercise condition in the CF diet.

(GPR41) (66, 67). Here, although the activation status of these receptors was not analyzed, a bout of intense exercise did not change the small intestine gene expression of FFAR2 and FFAR3. However, cocoa-enriched diet, but not cocoa-fiber diet, decreased the gene expression of FFAR3, which is mainly activated by propionic and butyric acids and is involved in energy balance maintenance, noradrenaline release from sympathetic neurons and the production of anorectic hormones like the peptide YY, among other functions (68). Then, these changes in the intestinal gene expression of FFAR3 may be somehow associated with the lower BW gain observed in C10 animals (and not in CF rats), here as well as in other studies from our research group (22, 33, 36) and from other authors (69, 70). Moreover, the anti-obesity potential of cocoa has already been discussed in several reviews (21, 71–73). Although we previously attributed the lower BW gain of rats fed with cocoa to the impact of theobromine and flavonoids on lipid and glucose metabolism, fat deposition and even the gut microbiota composition (22, 74), the current results suggest that a decrease in FFAR3 intestinal expression may also contribute to this multifactorial effect.

The intestinal epithelium, by means of the enterocytes, the tight junction proteins, the mucus layer and immune cells, plays an essential role as a barrier that regulates the permeability of nutrients, water and ions, and prevents the invasion of pathogenic bacteria, and together with the intestinal microbiota contributes to the maintenance of the intestinal homeostasis. Numerous studies have shown that intensive exercise impairs the expression and the phosphorylation status of the TJ proteins, disrupting the integrity of the intestinal epithelial barrier and leading to an increased gut permeability (15, 75). This impairment has been associated with the hyperthermia (76), dehydration (77) and the stress (78) induced by intensive exercise.

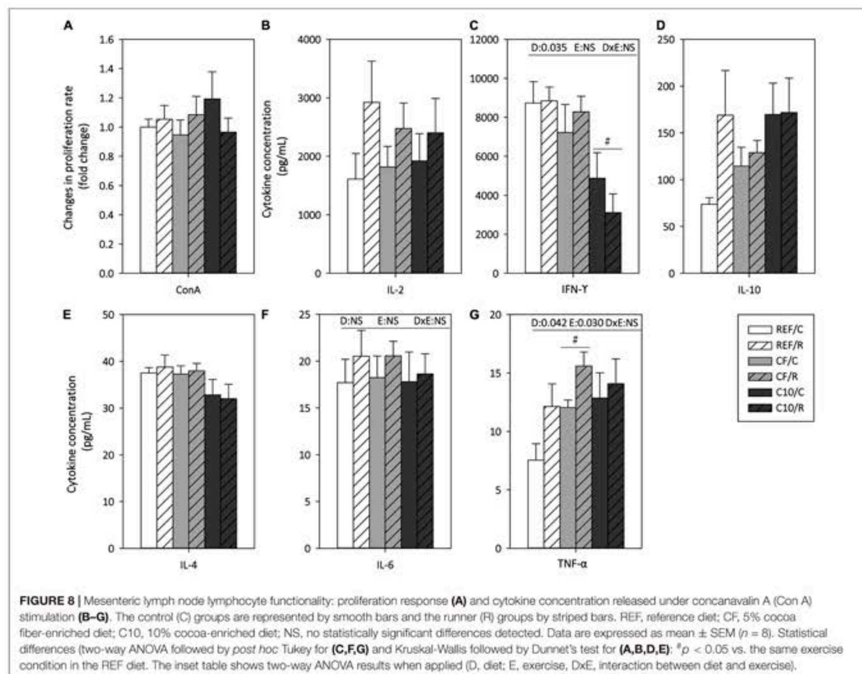

FIGURE 8 | Mesenteric lymph node lymphocyte functionality: proliferation response **(A)** and cytokine concentration released under concanavalin A (Con A) stimulation **(B–G)**. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet; NS, no statistically significant differences detected. Data are expressed as mean ± SEM ($n = 8$). Statistical differences (two-way ANOVA followed by *post hoc* Tukey for **(C,F,G)** and Kruskal-Wallis followed by Dunnet's test for **(A,B,D,E)**: $^{\#}p < 0.05$ vs. the same exercise condition in the REF diet. The inset table shows two-way ANOVA results when applied (D, diet; E, exercise, DxE, interaction between diet and exercise).

Lambert et al. associated an increased gastric and small intestinal permeability, with a 1.5% of BW loss in trained distance runners after running without fluid for 60 min at 70% VO_2 max (77). The level of dehydration described is like that we found in REF animals. Although we did not assess changes in intestinal permeability *per se*, we studied the effect of exercise on the small intestine gene expression of TJ proteins, which are essential for the regulation of gut paracellular permeability. The acute exercise protocol applied in the current study did not induce changes in Ocldn, Cldn4, ZO1 or ZO2 small intestine gene expression in REF animals. These results are in line with those of Chaves et al. (13), showing no changes in cldn1 and ZO1 intestinal expression of rats 12 h after performing acute exercise without prior training, although they observed a larger colonic villi interspace in runner rats. Nevertheless, Ducray et al. reported a lower ZO1 intestinal protein expression in rats 4 h after exhaustion running (79). On the other hand, cocoa diet, due to its fiber content, substantially increased the gene expression of ZO1, which agrees with studies recently published (80) and could be somehow associated with the protective effect of cocoa fiber on the increased GI motility induced by acute exercise. Taking together with the results regarding microbiota and intestinal epithelium, our results here suggest that cocoa consumption can improve intestinal health, especially by means of increasing cecal SCFA production and ZO1 expression, which could be useful not only to prevent the alterations induced by intensive exercise, but also in situations with an even more impaired intestinal barrier function, like some gastrointestinal disorders.

Whereas the study of exercise's impact on gut microbiota has just gained importance in recent years, its effect on IgA production has been widely studied. IgA is the main Ig isotype in the mucosal compartment and plays many essential roles, such as neutralization of pathogens and toxins, antigen sampling and blocking excessive commensal and pathogenic bacteria (55). It has been reported that regular sessions of moderate intensity exercise increase salivary IgA secretion whereas acute intensive exercise or prolonged periods of exhausting exercise might decrease it, explaining the higher rate of mucosal infections observed in athletes (81). Here, our protocol of acute exercise did not induce significant changes in IgA concentration in SMGs

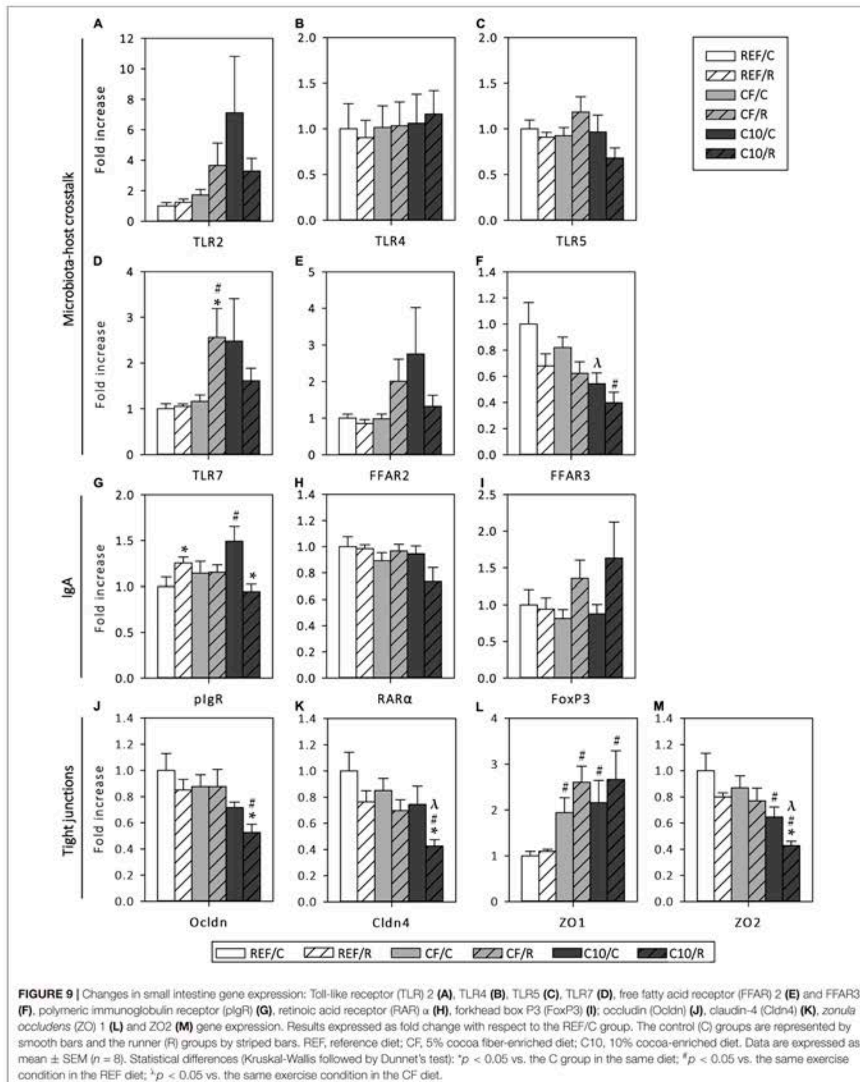

FIGURE 9 | Changes in small intestine gene expression: Toll-like receptor (TLR) 2 **(A)**, TLR4 **(B)**, TLR5 **(C)**, TLR7 **(D)**, free fatty acid receptor (FFAR) 2 **(E)** and FFAR3 **(F)**, polymeric immunoglobulin receptor (pIgR) **(G)**, retinoic acid receptor (RAR) α **(H)**, forkhead box P3 (FoxP3) **(I)**; occludin (Ocldn) **(J)**, claudin-4 (Cldn4) **(K)**, *zonula occludens* (ZO) 1 **(L)** and ZO2 **(M)** gene expression. Results expressed as fold change with respect to the REF/C group. The control (C) groups are represented by smooth bars and the runner (R) groups by striped bars. REF, reference diet; CF, 5% cocoa fiber-enriched diet; C10, 10% cocoa-enriched diet. Data are expressed as mean ± SEM ($n = 8$). Statistical differences (Kruskal-Wallis followed by Dunnet's test): $^{*}p < 0.05$ vs. the C group in the same diet; $^{\#}p < 0.05$ vs. the same exercise condition in the REF diet; $^{\lambda}p < 0.05$ vs. the same exercise condition in the CF diet.

or in the intestinal compartment, however, exercised rats had a lower concentration of salivary IgM, which has been described by others to be both decreased and increased after intensive exercise (12, 82). It must be taken into account that IgM is synthetized more quickly than IgA (83) and then it could be faster affected by an acute exercise. Moreover, it is important to clarify that whereas most of the published articles assessed changes in salivary secretory IgA, here we quantified the total concentration of IgA and IgM in submaxillary salivary gland homogenates, which may indicate changes in the ability to produce these antibodies, but not in the transcytosis and exocytosis processes. In further studies, we will ascertain the impact of acute exercise on salivary IgA and IgM concentrations, as well as how it affects their synthesis, exocytosis and transcytosis. Although the cocoa fiber diet, on its own, prevented this decrease in salivary IgM, the cocoa-enriched diet decreased it, both in the control and in exercised animals. These results are in line with results reported in healthy non-exercised rats (32). Moreover, in agreement with previous studies (36), the cocoa-enriched diet decreased the IgA concentration in SMGs. Therefore, cocoa diet, mainly due to bioactive compounds other than fiber, attenuated salivary Ig content. With regards to the intestinal compartment, animals from the CF and C10 groups had a lower concentration of IgA in GW after running.

In addition, to shed some light on the influence on mucosal immunity, we have studied the lymphocyte composition of two compartments of the gut-associated lymphoid tissue (GALT): the PPs and the MLNs. The GALT is the largest lymphoid tissue in the body and comprises the organized or inductor GALT, which includes PPs, MLNs and isolated lymphoid follicles, and the diffuse or effector GALT, which is formed by scattered intraepithelial (IEL) and lamina propria (LPL) lymphocytes. IgA-secreting plasma cells are mainly generated in PPs (84). It has been described that intense exercise, due to adrenergic mechanisms, induces a mobilization of splenic lymphocytes, mainly T cells, into secondary lymphoid organs such as PPs (6). In the current study, although we did not quantify the total number of cells, we did not observe any exercise-induced substantial change in the proportion of T and B cells in PPs, although it modified the proportion of the Tγδ cell subsets, favoring the presence of the Tγδ CD8αβ cells over that of Tγδ CD8αα cells in PPs. Tγδ CD8αβ cells have cytotoxic potential and secrete inflammatory cytokines, such as IFN-γ and TNF-α, that could contribute to the exercise-induced alterations in gut permeability that have been reported in other studies (37). On the other hand, the cocoa-enriched diet prevented the changes in Tγδ cell subsets in PPs, although it increased, as already described (85), the total proportion of Tγδ cells. These changes are not attributed to cocoa fiber content and may be due to other bioactive compounds such as polyphenols and methylxanthines. Nevertheless, cocoa diet, as already reported (85) increased the relative proportion of PPs Tc cells at the expense of Th cells, which could be partially associated with its fiber content.

The MLNs, together with the PPs, play an important role in initiating local immune responses in the gut (86). We found that, in this lymphoid organ, acute exercise induced an increase in NKT and Th cell proportions while it decreased that of Tc cells. These results agree with the reported higher Th/Tc cell ratio in MLNs of rats submitted to a 5-week intensive training (37). The results obtained here demonstrated that a single bout of exercise is able to change MLNLs proportions similarly to a 5-week training, probably due to differential mobilization of Th and Tc cells, but, on the contrary, while a 5-week training decreased the proliferative ability of T cells, the acute exercise applied here did not modify it. To our knowledge, there is no more available evidence on the effect of exercise on MLNs cell composition. Other authors have focused on brachial, axillary and submandibular lymph nodes, among others, but the results are not clear enough to draw any consistent conclusion (87). Cocoa fiber consumption, but not cocoa-enriched diet, prevented the changes induced by acute exercise in Th cells, suggesting the role of cocoa polyphenols or methylxanthines in counteracting the effect of cocoa fiber. On the other hand, both cocoa and cocoa fiber diets increased the proportion of Tγδ cells and decreased that of NKT cells. In the case of cocoa fed control animals, the higher proportion of Tγδ cells can be attributed to an increased proportion of Tγδ CD8αα cells, in agreement with a previous study (34).

Apart from these changes in T cell subset proportions in MLNs, acute exercise also modified their functionality, increasing the secretion of TNF-α, which could be explained by the cortisol released that occurs immediately after intensive exercise (29, 88, 89). Unlike what we previously observed in chronically intensively trained rats (29, 37), acute exercise did not manage to modify IL-2, IFN-γ, IL-4, and IL-6 production. On the other hand, although most of the studies reported a lower TNF-α production immediately after intensive exercise, normally involving a prior intensive training, due to the release of cortisol and noradrenaline (10, 90, 91), other authors have found no changes (13) or even an increased TNF-α production (92). Here, both acute exercise and cocoa fiber-enriched diet increased TNF-α secretion by *in vitro* stimulated MLNLs, in disagreement with previous studies showing an inhibitory effect on this cytokine production by cocoa consumption (20). Lastly, although our model of acute intensive exercise did not modify the IFN-γ levels released by stimulated MLNLs, the decrease induced by cocoa in the secretion of this proinflammatory cytokine could be useful to attenuate the substantial increase observed after a chronic intensive running training (37).

CONCLUSION

In conclusion, our results indicate that the intake of cocoa in the days prior to an intense bout of exercise can partially prevent the alterations induced by it. Some of the changes produced by the cocoa diet on the intestine can be attributed to its fiber content, but other changes denote interaction between the phenolic compounds and methylxanthines, which is worth studying depth in the future. Moreover, further research might ascertain whether a more intensive exercise or a sustained exercise can exacerbate the observed alterations, and assess the potential protective effect of cocoa fiber in an even more impaired immune system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee for Animal Experimentation of the University of Barcelona.

AUTHOR CONTRIBUTIONS

Under the supervision of MM-C and FP-C, PR-I completed these studies as part of her Ph.D. thesis. PR-I, MM-C, and FP-C contributed to the conception and design of the project. PR-I, MM-C, MR-L, AF, and MC-B performed the experimentation. PR-I conducted the literature search, the acquisition, analysis of data, and wrote the first version of the manuscript. All authors contributed to the revision of the manuscript, read, and approved the final work.

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ARTICLE 5

"Influence of hesperidin on systemic immunity of rats following an intensive training and exhausting exercise"

Patricia Ruiz-Iglesias, Sheila Estruel-Amades, Mariona Camps-Bossacoma, Malén Massot-Cladera, Francisco J. Pérez-Cano, Margarida Castell.

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ABSTRACT

Aim: To establish the influence of hesperidin supplementation on the immune alterations induced by intensive training and exhausting exercise in rats.

Methods: Female Wistar rats were randomized into two groups: runner and sedentary. The runner group undergone an intensive running training programme including three trainings per week and two exhaustion tests. Throughout the training period, 200 mg/kg of hesperidin or vehicle was administered by oral gavage three times per week. After 5 weeks of exercise and nutritional intervention, samples from the runner group were obtained either before, immediately after or 24 h after performing an additional exhaustion test to assess the influence of exercise on the immune status at different timepoints.

Results: Hesperidin supplementation improved the exercise performance during the chronic intensive training, avoiding the decrease in running capacity observed after the resting days in the non-supplemented rats. In addition, hesperidin prevented the increase in circulating leukocytes induced by the final exhaustion test and avoided the higher secretion of IFN-γ by peritoneal macrophages, although it did not prevented the increases in TNF-α and IL-6 production in exhausted rats.

Conclusion: Hesperidin supplementation by oral gavage three times per week improve exercise performance and attenuates the immune alterations induced by exercise training, alone or followed by a final session of exhausting exercise.

Article

Influence of Hesperidin on Systemic Immunity of Rats Following an Intensive Training and Exhausting Exercise

Patricia Ruiz-Iglesias [1,2], Sheila Estruel-Amades [1,2], Mariona Camps-Bossacoma [1,2], Malén Massot-Cladera [1,2], Àngels Franch [1,2], Francisco J. Pérez-Cano [1,2,*] and Margarida Castell [1,2,*]

1 Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), 08028 Barcelona, Spain; patriciaruiz@ub.edu (P.R.-I.); sheilaestruel@ub.edu (S.E.-A.); marionacamps@ub.edu (M.C.-B.); malen.massot@ub.edu (M.M.-C.); angelsfranch@ub.edu (À.F.)

2 Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), UB, 08921 Santa Coloma de Gramenet, Spain

* Correspondence: franciscoperez@ub.edu (F.J.P.-C.); margaridacastell@ub.edu (M.C.); Tel.: +34-93-402-45-05 (F.J.P.-C. & M.C.)

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Abstract: Intensive training and exhausting exercise can disrupt innate and acquired immunity. The flavanone hesperidin has shown immunomodulatory properties in physiological and some pathological conditions, and positive effects on exercise-induced oxidative stress. Nevertheless, it remains uncertain whether it also prevents exhausting exercise-induced immune alterations. The aim of this study was to establish the effect of oral hesperidin supplementation on the systemic immune system in rats following an intensive training and exhausting exercise. For this purpose, female Wistar rats were randomized into an intensive training group or a sedentary group. Intensive training was induced by running in a treadmill 5 days per week (including two exhausting tests) for five weeks. Throughout the training period, 200 mg/kg of hesperidin or vehicle was administered by oral gavage three times per week. At the end, blood, thymus, spleen and macrophages were collected before, immediately after and 24 h after an additional final exhaustion test. Hesperidin supplementation enhanced natural killer cell cytotoxicity and the proportion of phagocytic monocytes, attenuated the secretion of cytokines by stimulated macrophages, prevented the leukocytosis induced by exhaustion and increased the proportion of T helper cells in the thymus, blood and spleen. These results suggest that hesperidin can prevent exhausting exercise-induced immune alterations.

Keywords: blood; cytokines; exercise; exhaustion; lymphocytes; macrophages; NK cells; phagocytosis; spleen; thymus

1. Introduction

Nowadays, there is no doubt about the benefits of regular physical activity for health and in the prevention of several diseases. Sedentary life is a risk factor for cardiovascular disease and other chronic diseases, such as type 2 diabetes, cancer, osteoporosis, obesity, hypertension and depression [1,2]. Physical activity stimulates the body's cardiorespiratory, musculoskeletal and metabolic systems, making them more efficient. It is important to point out that the immune system functionality is also influenced by physical exercise. In comparison with sedentary behavior, it is well established that regular or moderate physical activity improves the immune system defensive function [3]. However, its functionality can be reduced when the exercise performance is extreme or excessive. In fact, acute

vigorous exercise alters almost all blood immune cells influencing their functional capacity [4–7]. As an example of detrimental effects of excessive physical activity, it has been reported that elite endurance athletes during periods of intensive training have a higher frequency of upper respiratory tract infections [4,8], similarly to the effects on non-elite runners following a marathon [9]. The damaging outcomes of physical exercise depend on its bout and load and these are still controversial [10]. Some data demonstrate that high training loads in athletes induce physiological alterations including, among others, impaired motor coordination, a decrease in muscle strength, a decrease in maximal oxygen uptake and endurance capacity [8], certain inflammatory status and oxidative stress [11], which all together can eventually result in decreased performance. We have recently demonstrated that intensive training and exhausting exercise in rats impairs innate immunity [6], in particular the phagocytic and the natural killer (NK) cytotoxic activities and macrophage cytokine secretion. This excessive exercise also has an impact on acquired immunity, inducing changes in the lymphocyte distribution and functionality [7].

In the field of nutritional support, there is interest in interventions that help to increase exercise performance, to reduce fatigue and to counteract the detrimental effects associated with extreme physical activity. For periods of intense physical activity, ensuring adequate energy, carbohydrate and protein intake as well as avoiding deficiencies of micronutrients are key strategies to avoid detrimental effects on the body and maintain immune health [12]. Among micronutrients, evidence is accumulating that supplements with flavonoids as well as other polyphenols can be useful for athletes. Flavonoids are products of plants secondary metabolism and are consumed through vegetables in the diet. In general, they are poorly absorbed in the human small intestine, reaching the colon where microbiota metabolizes them into products that can be absorbed and exert bioactive effects [13]. Flavonoids, as they are polyphenols, are able to improve antioxidant defenses and they are suggested to counteract strenuous exercise-associated oxidative stress and inflammation. In this context, it has been reported that consumption of quercetin for one or two weeks by untrained volunteers results in higher performance in bike-cycling [14] and treadmill training [15]. Likewise, consumption of a Montmorency cherry juice concentrate, containing high levels of flavonoids, improves the recovery of isometric muscle strength after intensive exercise in well-trained athletes [16].

Within the flavonoid family, citrus fruits mainly contain flavanones such as hesperidin. Besides its antioxidant capacity [17], hesperidin has demonstrated beneficial biological activities in systemic and intestinal immunity [18,19]. With regard to physical activity, it has been demonstrated that supplementation with a citrus flavonoid extract for 4 weeks improves cycling time-trial performance in trained male athletes [20]. In addition, rats receiving a hesperidin supplementation for 4 weeks and submitted to swimming, improved the biochemical profile and antioxidant biomarkers [21]. Moreover, hesperidin supplementation for 5 weeks in intensively trained rats prevented oxidative stress induced by intensive and exhausting exercise and improved exercise performance [22]. Based on this background, the current study aimed to ascertain the influence of hesperidin supplementation in the systemic immune system functionality in rats following an intensive training and exhausting exercise.

2. Materials and Methods

2.1. Animals

Sixty-four three-week-old female Wistar rats (Envigo, Huntingdon, United Kingdom) were maintained in polycarbonate cages containing bedding of large fibrous particles (Souralit 1035, Bobadeb S.L., Santo Domingo de la Calzada, Spain) (4 rats per cage) under controlled conditions (temperature and humidity, in a 12/12 h light/dark cycle) in the animal facilities of the Faculty of Biology at the University of Barcelona. Female rats were chosen because previous studies showed that this gender had better adaptability and higher performance than male rats [6]. Animal procedure was approved by the Ethical Committee for Animal Experimentation of the University of Barcelona and the Catalonia Government (CEEA/UB ref. 464/16 and DAAM 9257, respectively), in full compliance

with national legislation following the European Union (EU)-Directive 2010/63/EU for the protection of animals used for scientific purposes. The sample size required (N = 8/group) was determined by the Appraising Project Office's program from the Universidad Miguel Hernández de Elche (Spain), using the leukocyte total number for this calculation.

2.2. Diet and Hesperidin Supplementation

Water and food (Teklad Global 14% Protein Rodent Maintenance Diet, Teklad, Madison, WI, USA) were provided ad libitum throughout the study. Body weight (BW) and food intake were also monitored. Food efficiency was calculated by dividing the total BW gain in a cage by the total food intake of the same cage.

A half of animals, including both runner and sedentary groups, received a supplement of hesperidin (HealthTech BioActives, Murcia, Spain) administered by oral gavage three times per week at a dose of 200 mg/kg BW in 0.5% carboxymethylcellulose solution, prepared daily. This dosage was established due to its immunomodulatory effects evidenced in previous studies [18,19]. Non-supplemented rats received the same volume of the vehicle.

2.3. Exercise Training Program and Samples Collection

After a 7-day acclimation period, exercise training began by using two specialized treadmills for rodents: a LE8700 treadmill (Panlab, Harvard, USA) and an Exer3/6 treadmill (Columbus, OH, USA). The exercise training program has been previously described [6,7]. Briefly, all the 64 rats were firstly adapted to the treadmill for 10 days by increasing time and speed. At the end of this adaptation period, rats were randomly divided into four groups: non-supplemented runner animals (RUN, n = 24), hesperidin-supplemented runner animals (H-RUN, n = 24), non-supplemented sedentary animals (SED, n = 8) and hesperidin-supplemented sedentary animals (H-SED, n = 8). Thereafter, hesperidin supplementation began and both RUN group and H-RUN group were submitted to a 5-day period with increasing speed and duration followed by a 5 weeks' intensive training. In each week, rats carried out an exhaustion test every Monday and Friday, whereas on Tuesday, Wednesday and Thursday rats ran for a limited and increasing time according to the maximum speed achieved in the previous Monday's exhaustion test. Throughout the training program, SED and H-SED were exposed to the same conditions of isolation in a turned-off treadmill for the same period as runner rats. After each running session, runner rats received a solution of 50% condensed milk (100 µL/100 g BW) as a positive reward to reinforce their running. Sedentary animals also received this solution.

At the end of the 5-week training program, each runner group (RUN and H-RUN) was distributed into three subgroups, each one with a similar average in the ability to run, and two of these three groups carried out an additional final exhaustion test. The three subgroups allow the immune function assessment at different time points. For RUN and H-RUN animals, the groups were: Trained rats (T and H-T groups, n = 8 each one), which were euthanized 24 h after a regular training session, Trained with Exhaustion (TE and H-TE groups, n = 8 each one), which were euthanized immediately after carrying out an additional final exhaustion test, and Trained and after 24 h of Exhaustion (TE24 and H-TE24 groups, n = 8 each one), which were euthanized 24 h after the additional final exhaustion test.

Sedentary rats (both SED and H-SED groups) were euthanized randomly distributed over the 3 consecutive days. Animals were anesthetized with ketamine (90 mg/kg, Merial Laboratories S.A., Barcelona, Spain) and xylazine (10 mg/kg, Bayer A.G., Leverkusen, Germany). Peritoneal macrophages, blood from heart, thymus, and spleen were immediately collected and processed. Peritoneal macrophages allowed the determination of cytokine secretion after stimulation. Blood was used to determine leukocyte differential counts by an automated hematology analyzer (Spincell, MonLab Laboratories, Barcelona). Other blood samples were used for characterizing lymphocyte composition and for establishing the phagocytic activity. Lymphocytes from thymus and spleen were isolated and used for characterizing lymphocyte composition. From spleen lymphocytes,

the quantification of the proliferative response and the assessment of NK cell cytotoxic activity were also carried out.

2.4. Lymphocyte Composition in Blood, Thymus and Spleen

Thymus and spleen lymphocytes were isolated by smashing tissues in a sterile mesh cell strainer (40 μm) as previously described [7,23]. Splenocyte suspensions and blood samples were submitted to erythrocyte lysis. Afterwards, lymphocyte subsets were determined by mouse anti-rat CD161b, CD45RA, CD8α, CD8β, CD4, TCRαβ or TCRγδ antibodies (BD Biosciences, San Diego, CA, USA) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin, peridininchlorophylla protein, allophycocyanin or brilliant violet 421, as described previously [7,23]. Blood B cell proportion was approached by considering those lymphocytes negative for TCRαβ, TCRγδ and CD161b antibodies. In the thymus, the subsets considered were those bearing TCRαβ, which indicate mature thymocytes, and those expressing CD4 and/or CD8, which allow their classification in double negative (DN) cells (the most immature), double positive (DP) cells (the following state of maturation), and single positive cells, i.e., CD4+CD8- cells and CD4-CD8+ cells (the most mature thymocytes).

Data were acquired with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the Flow Cytometry Unit of the Scientific and Technological Centers of the UB (CCiTUB) and analyzed with FlowJo v.10 software (Tree Star, Inc., Ashland, OR, USA). The percentage of positive cells in the lymphocyte population selected was established according to forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) or in a particular lymphocyte population.

2.5. Phagocytic Activity

Blood monocyte and granulocyte phagocytic function was assessed by flow cytometry analysis using the Phagotest™ kit (Glycotope, Biotechnology GmbH, Heidelberg, Germany) in accordance with the manufacturer's instructions, as previously reported [6]. Data were acquired using Gallios™ Cytometer (CCiTUB) and the analysis was performed with FlowJo v.10 software. Monocyte and granulocyte subsets were selected according to their FSC/SSC. The percentage of phagocytic monocytes and granulocytes was quantified by means of the proportion of FITC+ cells, whereas their phagocytic activity was measured through mean fluorescence intensity (MFI).

2.6. Macrophage Cytokine Production

Peritoneal macrophages were collected by injecting cold phosphate-buffered saline (PBS) into the peritoneal cavity as previously reported [22]. Macrophages were suspended in cold Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL streptomycin-penicillin and 2 mM L-glutamine, plated into 12-well plates (10^6 cells/mL) and incubated for 2 h. After removing non-attached cells, macrophages were stimulated with 100 ng/mL lipopolysaccharide (LPS); non-stimulated macrophages were used as control. Supernatants were collected after overnight incubation. Interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α were quantified by using ProcartaPlex® Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, CA, USA), as previously described [21]. Data were acquired by Luminex MAGPIX analyzer (Luminex®) in the CCiTUB and analyzed with ProcartaPlex® Analyst (Thermo Fisher Scientific, S.L.U, Barcelona, Spain).

2.7. Natural Killer (NK) Cell Cytotoxic Activity

The cytotoxic activity of spleen NK cells was quantified by the NKTEST™ kit (Glycotope, Biotechnology GmbH, Heidelberg, Germany) following the manufacturer's protocol as previously reported [6]. Data were acquired by the Gallios™ Cytometer (CCiTUB) and the analysis was performed with FlowJo v.10 software. Spontaneous cell death (without effector cells) was considered as control. Results from the individual cytotoxic activity were calculated according to the total NK activity and the percentage of NK cells of each sample.

2.8. Spleen Lymphocyte Proliferation

Spleen lymphocytes (10^5 cells/well) were stimulated for 48 h, with either concanavalin A (ConA, 5 μg/mL, Sigma-Aldrich, Madrid, Spain) or pokeweed mitogen (PWM) (10 μg/mL, Sigma-Aldrich) to stimulate T and B cells, respectively. Assay was performed in quadruplicate and nonstimulated cells were maintained under the same conditions. Proliferative cells were quantified using a BrdU Cell Proliferation Assay kit (MerckMillipore, Darmstadt, Germany), as previously described [7,24]. Results are expressed as the percentage of cell proliferation increase after ConA and PWM stimulation with respect to unstimulated cells.

2.9. Plasma Cortisol Concentration

Plasma cortisol concentration was measured using DetectX® Cortisol enzyme-linked immunosorbent assay (ELISA, Arbor Assays, MI, USA) in accordance with the manufacturer's instructions. Absorbance was measured on a microplate photometer (Labsystems Multiskan, Helsinki, Finland) and data were interpolated by Ascent v.2.6 software (Thermo Fisher Scientific) according to the respective standard.

2.10. Statistical Analysis

Analysis of the data was carried out using IBM Social Sciences Software Program (SPSS, version 22.0, Chicago, IL, USA). The equality and normality of the data were tested by Levene's and Shapiro–Wilk's test, respectively. A two-way analysis of variance (ANOVA) test was applied and, if significant differences were detected, Tukey's post hoc test was performed. A Kruskal–Wallis test was used when results were neither equally nor normally distributed, followed by Mann–Whitney U test in the case of significant difference among groups. To compare variables during the study (e.g., changes in maximum time supported in the exhaustion tests), a repeated-measures ANOVA was applied followed by Student's *t*-test. Significant differences were considered when $p \leq 0.05$, except regarding repeated comparisons (Student's *t*-test), when p value was corrected (Bonferroni correction), dividing it by the number of applied tests.

3. Results

3.1. Effect of Hesperidin Supplementation on Food Efficiency and Training Performance

Body weight gain per rat (and the total per cage), and food consumption per cage were monitored throughout the study, allowing the calculation of the food efficiency. Both non-supplemented and hesperidin-supplemented runner animals showed higher food efficiency than the corresponding sedentary group and there were no significant effects due to hesperidin supplementation (Figure 1a).

Figure 1b summarizes the changes in maximum time achieved in each exhausting test performed every Monday and Friday throughout the study considering the first exhaustion test as 100%. The mean time supported in the first exhaustion test was 23.83 ± 0.51 min and 22.39 ± 0.58 min (mean ± SEM) for non-supplemented and hesperidin-supplemented animals, respectively. Throughout the study and in general, rats receiving hesperidin ran for longer periods in the exhaustion tests than non-supplemented rats. The hesperidin-supplemented group improved performance for the first 4 weeks (each subsequent Monday ran for longer time than the first Monday) and did not show the Monday's decreases in performance undergone by non-supplemented animals. Nevertheless, the improvement in physical performance due to hesperidin was not observed in the exhaustion tests carried out on the last three Fridays. At the end of the study, in the additional final exhaustion test, the maximum time supported by both hesperidin and non-hesperidin supplemented groups was similar (32.14 ± 1.83 min and 32.07 ± 1.50 min, respectively), reaching a mean speed of about 62 m/min in both groups. Similarly, plasma cortisol levels measured at the end of the study, which increased with exhaustion, did not change in hesperidin-supplemented rats (Figure 1c). Likewise, relative heart weight was higher in exhausted animals than in sedentary animals from both non-supplemented and supplemented

groups. However, 24 h later, while heart weight was completely restored in non-supplemented animals, it tended to remain higher in the hesperidin-supplemented group (p = 0.09 between H-SED and H-TE24) (Figure 1d).

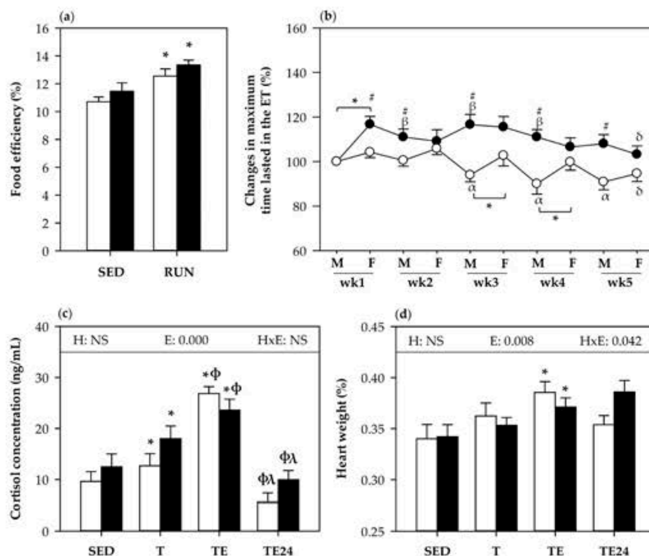

Figure 1. Food efficiency during the study (**a**), changes in maximum time supported in the exhaustion tests performed throughout the study (percentage with respect to the first exhaustion test) (**b**), cortisol concentration (**c**), and relative heart weight (**d**) at the end of the study. ET = exhaustion test; F = Friday; M = Monday; RUN = runner animals; SED = sedentary animals; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test; wk = week. NS = no statistically significant differences detected. The non-supplemented group is represented by white symbols (○) and bars, and the hesperidin-supplemented group by black symbols (•) and bars. Data are expressed as mean ± standard error of the mean (SEM, n = 3-8 for **a**, n = 24 for **b**, n = 8 for **c** and **d**). Statistical difference in (**a**,**c**,**d**), the inset table shows two-way analysis of variance (ANOVA) results when applied (H, hesperidin; E, exercise, H–E, interaction between hesperidin and exercise): * $p < 0.05$ vs. SED group; ϕ $p < 0.05$ vs. T group; λ $p < 0.05$ vs. TE group. Statistical difference in (**b**): * significant differences between consecutive ETs for both non-supplemented and the hesperidin-supplemented groups ($p < 0.05$); α significant differences in the non-supplemented group vs. the first and the second Monday ET ($p < 0.05$); β significant differences in the hesperidin-supplemented group vs. the first Monday ET ($p < 0.05$) and δ significant differences in both non-supplemented and hesperidin-supplemented groups vs. the first Friday ET (repeated-measures ANOVA followed by paired Student's t test); # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.005$, Student's t-test).

3.2. Blood Leukocytes Count and Lymphocyte Proportions

The counts of blood leukocytes and the proportion of lymphocytes, monocytes and granulocytes were assessed by an automatic hematology analyzer at the end of the study (Figure 2). Immediately after the final exhaustion test (TE group), blood leukocyte counts increased in non-supplemented rats

(Figure 2a). This increase was prevented by hesperidin supplementation (H-TE group). The percentages of lymphocytes, granulocytes and monocytes (Figure 2b–d) showed that the leukocytosis observed in the TE group was mainly due to lymphocytes because its percentage increased and that of granulocytes and monocytes decreased with respect to the T group.

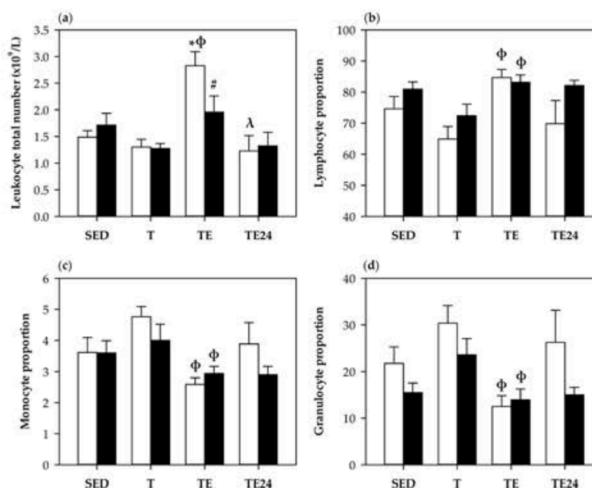

Figure 2. Blood leukocytes counts (**a**), and percentages of lymphocytes (**b**), monocytes (**c**) and granulocytes (**d**). The non-supplemented groups are represented by white bars and the hesperidin-supplemented groups by black bars. SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. Data are expressed as mean ± SEM (n = 8). Statistical difference (Mann–Whitney U test): * $p < 0.05$ vs. SED group; ϕ $p < 0.05$ vs. T group; λ $p < 0.05$ vs. TE group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

Flow cytometry analysis of blood lymphocytes allows the proportions of the main blood lymphocyte subsets to be established (Figure 3). Although trained rats (T group) did not significantly modify blood lymphocyte population distribution in comparison with the SED group, the final exhaustion test caused changes that were mainly observed when comparing with the T group.

The lymphocytosis observed by hematology analyzer was due to a tendency to increase the T cell proportion in the TE group when compared with the T group (p = 0.1, TE group vs. T group). A similar tendency was observed in the exhausted rats receiving hesperidin (Figure 3a). When considering the Th and Tc cell subsets in T lymphocytes, the proportion of Th cells increased and that of Tc cells decreased in the TE group (p = 0.05 vs. T group), indicating that Th lymphocytes were mainly involved in the lymphocytosis induced by the exhausting exercise (Figure 3f–g).

One day after the final exhaustion test, the non-supplemented animals (TE24 group) showed a decrease in the T lymphocyte percentage (p = 0.049 vs. T group, p = 0.009 vs. TE group). On the contrary, hesperidin supplementation kept a higher T cell proportion in this group (p = 0.049, H-TE24 group vs. TE24 group), mainly due to a higher percentage in the Th cell subset (p = 0.046, H-TE24 group vs. TE24 group).

Blood NK, NKT (NK with TCRαβ+) and TCRγδ+ cell proportions decreased immediately after exhaustion, nevertheless, hesperidin supplementation tended to attenuate the reduction of NK and

NKT cells ($p = 0.063$ and $p = 0.085$ between the TE group and H-TE group, respectively). The percentage of TCRγδ+ cells did not change due to hesperidin supplementation (Figure 3c–e).

In addition, one week before the end of the study, the proportion of blood regulatory T cells (Treg) and activated Th cells (Tact) were evaluated (Figure 3h–i). The Treg percentage was not modified by exercise. However, the proportion of Tact cells in blood was lower in hesperidin-supplemented SED animals than in the non-supplemented counterpart group ($p = 0.010$).

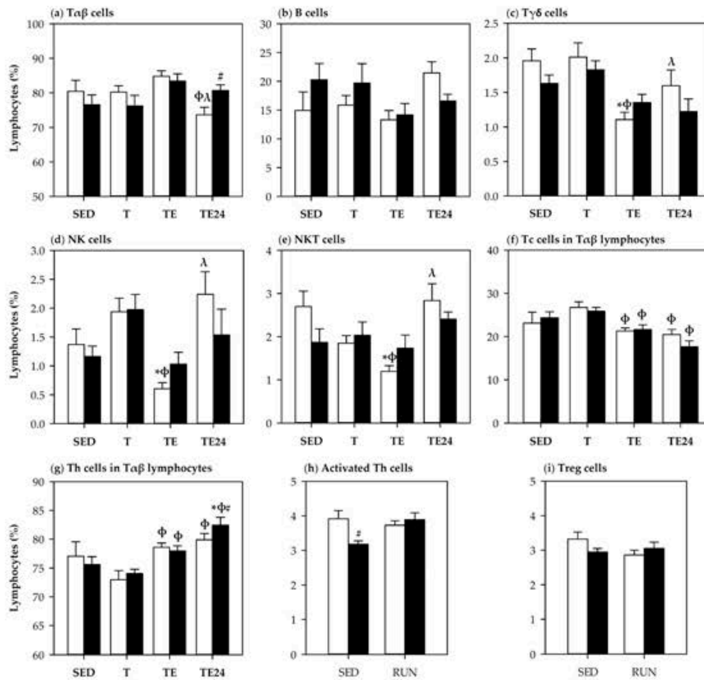

Figure 3. Percentages of blood lymphocytes: T (TCRαβ+) (**a**), B (TCRαβ- TCRγδ-CD161b-) (**b**), Tγδ (TCRγδ+) (**c**), natural killer (NK) (CD161b+) (**d**), natural killer T (NKT) (CD161b+TCRαβ+) (**e**), Th (CD4+CD161b- in TCRαβ+) (**f**), Tc (CD8+CD161b- in TCRαβ+) (**g**), Tact (CD25+Foxp3- in CD4+ TCRαβ+) (**h**), and Treg (CD25+Foxp3+ in CD4+ TCRαβ+) (**i**) cells. The proportions of Treg and Tact cells were established one week before final exhausting test (therefore, all runners). The non-supplemented groups are represented by white bars and the hesperidin-supplemented groups by black bars. SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. Data are expressed as mean ± SEM ($n = 8$ for **a**–**g**, $n = 8$–15 for **h**,**i**). Statistical difference (Mann–Whitney U test): * $p < 0.05$ vs. SED group; ϕ $p < 0.05$ vs. T group; λ $p < 0.05$ vs. TE group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

3.3. Lymphocyte Composition in Thymus and Spleen

Changes in thymus lymphocyte composition due to exhausting exercise and hesperidin supplementation were also assessed (Table 1). Just training did not significantly modify the thymocyte subsets studied here. Nevertheless, after running the final exhaustion test, some changes appeared when compared with trained rats. Non-supplemented animals showed a lower proportion of TCRαβ+ cells immediately after carrying out the additional final exhaustion test (TE group) than the other conditions ($p = 0.050$ vs. SED group, $p = 0.022$ vs. T group), which was accompanied by a tendency to decrease the percentage of CD4+CD8- cells ($p = 0.064$, TE group vs. T group) and to increase that of CD4-CD8+ cells ($p = 0.064$, TE group vs. T group). One day later, there was a notable increase in TCRαβ+ ($p = 0.000$, TE24 group vs. TE group) and CD4-CD8- ($p = 0.049$ vs. T group) cell proportions. These changes were also observed in hesperidin-supplemented groups. However, it is noteworthy that hesperidin supplementation decreased the relative proportion of CD4-CD8+ thymocytes in the H-TE group with respect to the TE group ($p = 0.003$) and showed a higher proportion of the CD4+CD8- subset 24 h later ($p = 0.018$, TE24 group vs. H-TE24 group).

Table 1. Percentages of thymus lymphocytes: TCRαβ+; double negative (DN) (CD4-CD8-); double positive (DP) (CD4+CD8+); mature TCD4+ (CD4+CD8-); and mature TCD8+ (CD4-CD8+) cells.

Thymus Lymphocyte Composition					
	Hesperidin	**SED**	**T**	**TE**	**TE24**
% in total lymphocytes:					
TCRαβ+	-	12.35 ± 1.27	14.94 ± 1.34	10.19 ± 0.74 $^{\phi}$	15.76 ± 1.13 $^{\lambda}$
	+	15.11 ± 1.42	12.72 ± 0.64	9.23 ± 0.98	17.31 ± 1.42 $^{\lambda}$
DN	-	3.62 ± 0.69	3.15 ± 0.45	2.82 ± 0.11	4.48 ± 0.43 $^{\lambda}$
	+	3.90 ± 0.52	3.15 ± 0.35	2.24 ± 0.31	4.44 ± 0.38 $^{\lambda}$
DP	-	81.85 ± 2.59	81.71 ± 1.88	84.11 ± 0.76	80.46 ± 1.48
	+	81.71 ± 1.06	84.51 ± 1.06	86.73 ± 1.59	76.5 ± 2.21
TCD4+	-	8.90 ± 1.47	8.93 ± 0.91	6.62 ± 0.37	8.21 ± 0.81
	+	10.36 ± 0.82	7.99 ± 0.71	5.93 ± 1.34	12.57 ± 0.98
TCD8+	-	5.62 ± 0.51	4.61 ± 0.61	6.25 ± 0.29	5.86 ± 0.39
	+	4.53 ± 0.41	4.33 ± 0.32	4.11 ± 0.19 $^{\#}$	6.16 ± 0.37

SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. - = non-supplemented groups; + = hesperidin-supplemented groups. Data are expressed as mean ± SEM ($n = 8$). Statistical difference (Mann–Whitney U test): ϕ $p < 0.05$ vs. T group; λ $p < 0.05$ vs. TE group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

Spleen lymphocyte composition was also studied (Table 2). Intensive training did not change the main lymphocyte subsets, i.e., B, T, Th and Tc cells, but did change the proportion of TCRγδ+ cells and NK cells, which decreased 24 h after the final exhaustion test ($p = 0.004$, TE24 group vs. SED group for TCRγδ+ cells; $p = 0.05$ vs. T group for NK cells). Hesperidin supplementation increased the proportion of T cells 24 h after the exhaustion test ($p = 0.018$, TE24 group vs. H-TE24 group).

3.4. Spleen Lymphocyte Proliferation

The functionality of spleen T and B cells was also assessed by their proliferation after ConA and PWM stimulations, respectively (Figure 4). The proliferation capacity of T cells was not modified by training but increased immediately after exhaustion (TE group) in both non-supplemented and hesperidin-supplemented animals ($p < 0.05$ vs. SED group and T group) (Figure 4a). One day after the final exhaustion test, T cell proliferation rate decreased in comparison with the TE group ($p = 0.022$, TE24 group vs. TE group) but remained higher than in the T group ($p = 0.010$, TE24 group vs. T group).

Table 2. Percentages of spleen lymphocytes: T (TCRαβ+); B (CD45RA+); Tγδ (TCRγδ+); NK (CD161b+); NKT (CD161b+ TCRαβ+); Th (CD4+CD161b- in TCRαβ+); and Tc (CD8+CD161b- in TCRαβ+) cells.

Spleen Lymphocyte Composition					
	Hesperidin	**SED**	**T**	**TE**	**TE24**
% in total lymphocytes:					
T	-	41.68 ± 1.72	40.41 ± 5.49	43.71 ± 2.68	43.24 ± 1.84
	+	47.07 ± 2.13	45.47 ± 1.89	43.51 ± 2.64	50.91 ± 1.16 #
B	-	36.83 ± 1.63	32.06 ± 2.06	38.10 ± 2.20	37.24 ± 1.97
	+	33.35 ± 1.96	34.46 ± 2.18	37.60 ± 1.62	32.41 ± 1.21
Tγδ	-	3.95 ± 0.22	3.32 ± 0.48	3.54 ± 0.26	2.89 ± 0.20 *
	+	3.64 ± 0.24	3.24 ± 0.24	3.44 ± 0.12	2.78 ± 0.22 *
NK	-	7.92 ± 1.05	6.21 ± 0.90	5.28 ± 0.51	5.01 ± 0.80 Φ
	+	6.43 ± 0.41	6.51± 1.13	5.70 ± 0.84	4.10 ± 0.41 Φ
NKT	-	4.71 ± 0.80	3.29 ± 0.49	3.48 ± 0.22	3.21 ± 0.29
	+	3.50 ± 0.17	4.24 ± 0.67	3.10 ± 0.23	3.33 ± 0.30
% in T lymphocytes:					
Th	-	74.88 ± 0.57	72.19 ± 1.09	74.09 ± 0.74	74.01 ± 0.92
	+	72.59 ± 1.66	70.00 ± 3.26	74.43 ± 0.38	75.34 ± 0.46
Tc	-	27.87 ± 1.27	28.57 ± 0.70	27.23 ± 0.85	26.60 ± 0.74
	+	28.21 ± 1.95	30.36 ± 2.73	27.71 ± 0.47	26.95 ± 1.90

SED = sedentary groups, T = trained groups, TE = T groups with an additional exhaustion test, TE24 = TE groups 24 h after the exhaustion test. - = non-supplemented groups; + = hesperidin-supplemented groups. Data are expressed as mean ± SEM (n = 8). Statistical difference (Mann-Whitney U test): * $p < 0.05$ vs. SED group; Φ $p < 0.05$ vs. T group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

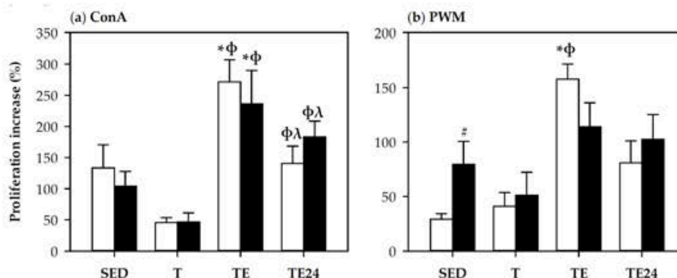

Figure 4. Proliferation capacity of spleen T lymphocytes (**a**) and B lymphocytes (**b**). The non-supplemented groups are represented by white bars and the hesperidin-supplemented groups by black bars. SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. Data are expressed as mean ± SEM (n = 8). Statistical differences (Mann–Whitney U test for **a**, and two-way ANOVA followed by Tuckey for **b**): * $p < 0.05$ vs. SED group; ϕ $p < 0.05$ vs. T group; λ $p < 0.05$ vs. TE group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

B cell proliferative capacity increased after exhaustion in non-supplemented animals ($p = 0.009$, TE group vs. SED group, $p = 0.006$, TE group vs. T group) (Figure 4b), nevertheless, hesperidin supplementation tended to attenuate this effect ($p = 0.1$, TE group vs. H-TE group). Moreover, hesperidin enhanced B cell proliferative ability in sedentary animals ($p = 0.038$, SED group vs. H-SED group).

3.5. NK Cell Cytotoxic Activity

The cytotoxic function of spleen NK cells was determined (Figure 5). In both non-supplemented and hesperidin-supplemented animals, there was a higher cytotoxic activity in all intensively trained and exhausted rats ($p < 0.05$ between SED groups and T, TE, and TE24 groups). Moreover, hesperidin increased the NK cytotoxic activity in sedentary animals ($p = 0.000$, SED group vs. H-SED group) and tended to raise that of the TE24 group ($p = 0.09$, TE24 group vs. H-TE24).

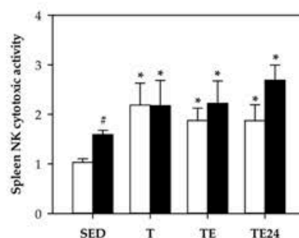

Figure 5. Spleen NK cytotoxic activity (number of dead target cells per 100 effector cells). The non-supplemented groups are represented by white bars and the hesperidin-supplemented groups by black bars. SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. Data are expressed as mean ± SEM ($n = 8$). Statistical difference (Mann–Whitney *U* test): * $p < 0.05$ vs. SED group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

3.6. Phagocytic Activity

The proportion of blood monocytes with phagocytic ability, as well as their phagocytic activity was assessed at the end of the study (Figure 6). The percentage of phagocytic monocytes was influenced both by intensive training and hesperidin supplementation (Figure 6a). In particular, 24 h after the final exhaustion test, the proportion of phagocytic monocytes increased ($p = 0.004$, TE24 group vs. SED, T and TE groups). This proportion was also enhanced by hesperidin supplementation immediately after carrying out the final exhaustion test ($p = 0.006$, TE group vs. H-TE group). Concerning the functionality of these monocytes, cells from the T group showed a lower phagocytic activity than those from the SED group ($p = 0.037$). Moreover, the increases in monocyte proportions 24 h after the exhaustion test were associated with a higher phagocytic activity in both hesperidin and non-hesperidin groups ($p < 0.05$, TE24 group vs. SED, T and TE groups) (Figure 6b).

No changes in the proportion of phagocytic granulocytes and their activity were observed due to training, exhaustion test or hesperidin supplementation (data not shown).

3.7. Macrophage Cytokine Production

The production of IFN-γ, IL-1β, IL-6, TNF-α, and IL-10 was quantified in supernatants of LPS-stimulated peritoneal macrophages obtained from sedentary and runner rats supplemented or not with hesperidin (Table 3).

Concerning IFN-γ secretion, in non-supplemented rats, intensive training (T group) was accompanied by a higher IFN-γ secretion than SED animals ($p = 0.014$), which was prevented by the hesperidin supplementation ($p = 0.05$, H-T group vs. T group). On the other hand, hesperidin supplementation induced a higher IFN-γ production in sedentary animals ($p = 0.042$, SED group vs. H-SED group) and tended to increase its levels 24 h after the final exhaustion test ($p = 0.076$, TE24 group vs. H-TE24 group).

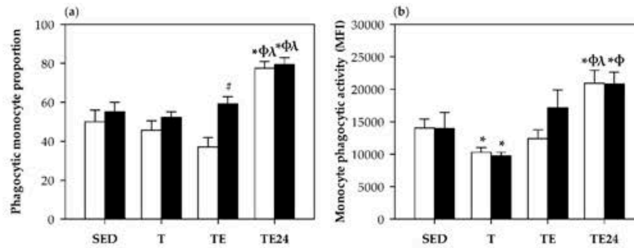

Figure 6. Phagocytic monocyte proportion (**a**) and monocyte phagocytic activity expressed as mean fluorescence intensity (MFI) (**b**). The non-supplemented groups are represented by white bars and the hesperidin-supplemented groups by black bars. SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. Data are expressed as mean ± SEM (n = 7–8). Statistical difference (Mann–Whitney U test): * $p < 0.05$ vs. SED group; ϕ $p < 0.05$ vs. T group; λ $p < 0.05$ vs. TE group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

Table 3. Cytokine concentration (pg/mL) released by peritoneal macrophages stimulated by LPS.

Peritoneal Macrophage Cytokine Secretion					
	Hesperidin	SED	T	TE	TE24
IFN-γ	-	28.14 ± 10.33	161.36 ± 34.75 *	46.01 ± 8.64 Φ	34.92 ± 15.00 Φ
	+	63.12 ± 11.19 #	46.92 ± 19.32 #	33.48 ± 10.40	124.6 ± 55.87
IL-1β	-	131.7 ± 23.01	102.7 ± 23.15	165.3 ± 12.05	141.7 ± 31.66
	+	127.6 ± 18.21	90.29 ± 16.41	147.1 ± 37.84	168.8 ± 21.69
IL-6	-	710.6 ± 167.3	1282.5 ± 155.4	1635.0 ± 224.6 *	1080.9 ± 316.5
	+	1054.0 ± 192.2	1032.2 ± 222.5	1358.7 ± 334.9	1737.2 ± 268.3
TNF-α	-	304.6 ± 76.60	146.8 ± 38.30	408.5 ± 63.30 Φ	359.2 ± 86.57 Φ
	+	350.0 ± 54.97	238.9 ± 56.38	353.9 ± 73.59	419.0 ± 51.32
IL-10	-	83.41 ± 19.73	97.22 ± 16.57	58.02 ± 28.21	87.03 ± 17.78
	+	118.7 ± 27.28	47.40 ± 8.74 #	54.48 ± 15.14	76.70 ± 27.53

SED = sedentary groups; T = trained groups; TE = T groups with an additional exhaustion test; TE24 = TE groups 24 h after the exhaustion test. − = non-supplemented groups; + = hesperidin-supplemented groups. Data are expressed as mean ± SEM (n = 5–8). Statistical difference (Mann–Whitney U test): * $p < 0.05$ vs. SED group; Φ $p < 0.05$ vs. T group; # significant differences between non-supplemented and the counterpart hesperidin-supplemented group ($p < 0.05$).

The secretion of IL-1β was not significantly modified by either intensive training or hesperidin supplementation.

IL-6 secretion increased by exercise, but there was only a significant effect just immediately after exhaustion ($p = 0.018$, SED group vs. TE group). Hesperidin-supplemented trained animals kept the IL-6 secretion closer to the values obtained in their sedentary counterpart animals (H-SED group).

TNF-α secretion increased immediately after exhaustion as well as 24 h later, when compared to the levels in trained rats ($p = 0.05$ and $p = 0.048$ T group vs. TE and TE24 groups, respectively). Hesperidin supplementation did not modify the TNF-α secretion profile.

IL-10 secretion was not altered by training or exhaustion but changed due to hesperidin supplementation. The levels of IL-10 in trained rats were lower in those animals receiving hesperidin than in those non-supplemented ($p = 0.002$, T group vs. H-T group).

4. Discussion

Previous studies demonstrated that rats submitted to intensive training and exhausting exercise exhibited alterations in both innate and acquired immunity [6,7]. In the current study we show the potential role of hesperidin supplementation in preventing some of these modifications.

First of all, as already reported [22], hesperidin supplementation partially improved exercise performance throughout the continuous training, avoiding the decrease in running capacity observed in the non-supplemented animals on Monday after resting for the weekend. Nevertheless, this effect was not observed in the exhaustion tests from the last three Fridays, and the additional final exhausting test, hence both non-supplemented and hesperidin-supplemented groups evidenced a decline in physical performance probably due to the high frequency of training and the lack of an adequate recovery between training sessions. The performance increase by hesperidin observed in the first weeks agrees with those reported in the cycling time-trial performance in trained male athletes after the intake of a citrus flavonoid extract [20], and those observed in healthy male amateur cyclists who carried out a repeated sprint test on a cycle ergometer after the acute intake of 500 mg of 2S-Hesperidin (Cardiose®) [25]. This increase in performance could be related to the antioxidant effect of hesperidin. In this context, rats receiving hesperidin and submitted to swimming or running, improved the antioxidant biomarkers and exercise performance [21,22]. Nevertheless, it has been highlighted that exercise-induced oxidative stress has been postulated as a positive regulator of the adaptation of endurance training which could explain that at the end of the study hesperidin supplementation did not produce a better performance [26]. Moreover, the lack of effect at the end of the study could be due to hesperidin inhibitory effects on prostaglandin synthesis [27,28]. In this context, the decrease of prostaglandin synthesis by anti-inflammatory drugs has been associated with an inhibition of strength and muscle hypertrophic adaptations [29,30]. In any case, it seems that hesperidin supplementation could be useful in increasing exercise performance in some cases and further studies should be carried out to establish the optimal dosage for these effects.

Considering the innate immunity, we have previously reported that a similar exhausting exercise reduced the spleen NK cell proportion but increased its cytotoxic activity, decreased the phagocytic activity by blood phagocytes, and altered the pattern of peritoneal macrophage cytokine secretion [6]. In the current study, the final exhaustion test decreased the blood percentage of the minor subsets NK, NKT and TCR$\gamma\delta$+ cells, and 24 h later decreased the proportion of NK cells in the spleen. Nevertheless, training and exhaustion were accompanied by an increase in cytotoxic activity, which agrees with previous results reported about NK cell proportion and activity [6,7,31]. Herein hesperidin supplementation tended to avoid the decrease in blood NK and NKT cell proportion and was able to increase the cytotoxic activity of NK cells, although this effect only reached statistical significance in sedentary animals. Although these results must be confirmed with further experiments, they could suggest a protective effect of hesperidin in NK cell function involved in the innate immune response and modified by intensive training. In this regard, an in vitro study shows that hesperidin increased spleen NK cell function, as well as the activity of splenic cytotoxic T lymphocytes [32]. Moreover, it has been described that daily anthocyanin consumption for 6 weeks prevented the decrease in blood NK cell count induced by 2.5 h of running by well-trained subjects [33]. Similarly, the enhancing effect of polyphenols, such as myricetin and resveratrol, among others, on NK cell activity has been reported [34–36]. Nevertheless, a study including the daily intake of 292 mg of hesperidin did not change blood NK cell proportion or their cytotoxic activity in healthy well-nourished humans [37]. Although there is some evidence, further studies focused on the effect of hesperidin on NK activity when there is a viral infection after intensive training, where these cells have a defensive role, must shed some light on the effect of this flavanone on NK cells. In this context, hesperidin has been considered as a potential protective agent against various infectious factors [38].

With regard to phagocytosis, our results showed that the proportion of blood phagocytic monocytes increased 24 h after exhaustion as did their phagocytic activity, in agreement with previous studies [6,39]. The hesperidin supplementation increased the proportion of blood phagocytic monocytes, which was

more evident immediately after exhaustion, in consonance with other studies that have suggested the helpful effect of this flavanone in restoring to normal levels the phagocytic index of neutrophils in Wistar rats after a toxic insult administration [40], and in increasing in vitro lysosomal phosphatase activity of macrophages, which may correlate to degranulation in phagocytosis [32]. Here, this enhancing effect could help to counteract some of the immediate immunodepressant effects of exhaustion, as phagocytosis is a key process in fighting against potential pathogenic invaders.

Intensive training and exhaustion also affect cytokine secretion by LPS-stimulated peritoneal macrophages. Previous studies demonstrate attenuations in the pro-inflammatory cytokine secretion, such as TNF-α and IL-12, with intensive training, which were overcome after exhaustion [6]. Here, we found no significant effects on the levels of TNF-α and IL-1β after training but there was an increase in TNF-α secretion after exhaustion, thus evidencing an inflammatory status induced by this high-load exercise. Moreover, there was an increase in IL-6 secretion by exhaustion, as previously reported in trained and exhausted rats [6] and also reported in plasma due to the secretion of muscle fibers [41–43]. In this case, hesperidin did not play a significant preventive effect. This agrees with a study reporting that daily hesperidin supplementation (100 mg/kg BW) did not restore the higher IL-6 secretion induced by high-fat diet intake in Wistar rats [44]. Nevertheless, other studies show that crude polyphenols extracted from blossoms of *Citrus aurantium* L. var. *amara* Engl., including neohesperidin and hesperitin, displayed an inhibitory effect on secretion of IL-6, TNF-α and IL-1β by murine macrophage RAW264.7 cell line in a concentration-dependent manner [45]. On the one hand, the secretion of IFN-γ increased remarkably with intensive training in peritoneal macrophages from trained rats and, interestingly, hesperidin supplementation prevented this effect in accordance with the inhibitory effect of hesperidin on IFN-γ previously described in an in vitro study using human mesenchymal stem cells [46] and in a mouse model of fulminant hepatitis [47]. However, in sedentary conditions, also 24 h after exhaustion, hesperidin increased (or tended to increase) the secretion of IFN-γ by peritoneal macrophages, suggesting its enhancing effect on innate immunity. Finally, in trained rats, hesperidin supplementation decreased the secretion of IL-10, a typical cytokine from M2 macrophages. It has been reported that polyphenols tend to polarize macrophages towards the M2 phenotype due to their anti-inflammatory properties [48]. However, in the current study, after performing an exhausting exercise the immune function is impaired, and hesperidin, as immunomodulator, may enhance macrophages' pro-inflammatory functions in order to decrease the risk of potential infectious diseases. Our results partially agree with those reported by Dourado et al. [49], showing a lower secretion of IL-10 by LPS-stimulated macrophages from non-exercised mice treated orally with hesperidin for 2 weeks, and also by in vitro studies in mesenchymal stem cells [46]. However they disagree with another in vitro study showing a higher expression of IL-10 in the RAW264.7 cell line after hesperidin incubation [50]. Thus, although the effect of polyphenols in general, and hesperidin in particular, in some macrophage functions such as production of reactive species of nitrogen and oxygen have been widely studied [22,48], the impact of hesperidin in macrophage phagocytosis, cytokine secretion and phenotype polarization in both healthy and immunosuppressed conditions remains controversial and must be elucidated in future studies.

Besides the effects of exercise and hesperidin in the innate immunity, some approaches in acquired immunity have been carried out. As observed in previous studies [6], an exhausting test in intensively trained animals induced an immediate leukocytosis that was overcome 24 h later. The leukocytosis was due to an increase in the lymphocyte counts. This fact has been associated with the increase of adrenalin secretion by exhaustion, and its ability to release leukocytes from the marginal compartment [51]. The analysis of blood lymphocytes by flow cytometry revealed that exhaustion was accompanied by an increase in Th cell proportion, as previously reported [7], and is in agreement with the fact that T cells mobilize faster than other lymphocytes from the spleen compartment [52]. Interestingly, hesperidin supplementation prevented the leukocytosis in exhausted rats. This could be due to an attenuating effect on the catecholamine release, although hesperidin was not able to decrease the cortisol hormone released by exhaustion as observed here. In addition, hesperidin attenuated the proportion of blood

T-activated lymphocytes in sedentary animals, which could be a reflection of its modulatory effect on the acquired immunity. Nevertheless, previous studies performed in healthy humans showed that hesperidin consumption induced no changes in T lymphocyte activation [37].

When considering the thymus, we observed that exhaustion reduced the proportion of the mature cells (TCRαβ+ cells and showed a tendency to decrease the CD4+CD8- subset), probably indicating the mobilization of these cells to the blood. Such decrease in TCRαβ+ cells was not prevented by hesperidin but, interestingly, its supplementation increased the CD4+CD8- proportion 24 h after exhaustion. These effects would suggest an enhanced effect of the flavanone on Th cell maturation. On the other hand, spleen T (Th and Tc) and B lymphocyte proportions were not modified by either intensive training or the final exhaustion test. Nevertheless, the administration of hesperidin was able to increase the relative proportion of T cells as significantly observed 24 h after the final exhaustion test (and with a tendency in the sedentary group). Although they do not match with those observed in blood, the results agree with the increase in CD4+CD8- thymocytes and also with results reported in mesenteric lymph nodes [18,19], which could be due to a more intense effect of hesperidin on lymphoid tissues.

Not only was lymphocyte composition modified by exhausting exercise, but also their functionality as it was quantified by means of their proliferation capacity. As previously reported [7], the proliferation of spleen T and B lymphocytes increased immediately after exhaustion. Moreover 24 h later, there was an increase in NK cytotoxicity and monocyte phagocytic activity, which partially disagrees with recent publications claiming that exercise may induce only a transient redistribution of immune cells at 1–2 h following a bout of intensive training, returning to baseline within 24 h [10]. On the other hand, in sedentary animals, we found a higher B cell proliferation capacity and no changes in that of T cells due to hesperidin supplementation. Nevertheless, we did not observe a marked protective effect of hesperidin on exhaustion-induced cell proliferation changes, only a tendency to normalize that of B cells. Although these results must be confirmed with future experiments, they would partially agree with previous studies that showed an increase in both T and B cell proliferation of murine splenocytes after 24 h in vitro stimulation with hesperidin, both in the absence of mitogen and after 48 h LPS or lectin-stimulation [32]. However, there is also evidence of the antiproliferative properties of some polyphenols in cancer [53] and, therefore, further studies would be necessary to elucidate the controversial effects of these bioactive compounds on cell proliferation in both overactivated and suppressed immune system conditions.

5. Conclusions

In summary, intensive training and an additional exhaustion test modify both innate and acquired immunity in rats. Here we demonstrate that the administration of hesperidin during training prevents the decrease in performance after a two-day resting period and enhances some aspects of the immune response. In some conditions, hesperidin is able to enhance the cytotoxic function of NK cells as well as the proportion of phagocytic cells, whereas it has an attenuating effect on some cytokines secreted by macrophages. Moreover, hesperidin prevents the leukocytosis induced by exhaustion and promotes the abundance of T (Th) cells in the thymus, blood and spleen, which can be observed, above all, the day after the exhaustion test. These immunoenhancing effects of this flavanone in intensive exercise may be confirmed and completed with further experiments. Such experiments should be focused on ascertaining whether the induction of an infectious process after training and exhaustion can be attenuated by the hesperidin administration.

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ARTICLE 6

"Dietary interventions with cocoa and hesperidin on the systemic immunity of intensively trained and exhausted rats"

Patricia Ruiz-Iglesias, Malén Massot-Cladera, Francisco J. Pérez-Cano, Margarida Castell.

Submitted

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- **XV Congrés de la Societat Catalana d'Immunologia.** Virtual, November 2021. Ruiz-Iglesias P.; Azagra-Boronat I.; Grases-Pintó B.; Massot-Cladera, M.; Castell, M.; Pérez-Cano, FJ. "**Effect of cocoa and orange polyphenols on cytotoxic and phagocytic activities of rats following an intensive training and exhausting exercise**".
- **III Jornada Catalana de Recerca en Ciències de l'Activitat Física i l'Esport.** Vic, June 2021. Gorgori-González, A.; Ruiz-Iglesias, P.; Castell, M.; Pérez-Cano, F. "**Estudio preclínico de la relación entre la ingesta de una dieta rica en cacao y hesperidina sobre el rendimiento deportivo, el ejercicio físico extenuante y su respuesta inmunitaria**".

ABSTRACT

Aim: To establish the potential synergistic effects between cocoa and hesperidin in intensively trained and exhausted Lewis rats.

Methods: Lewis rats were fed either a standard diet, a diet containing 10% cocoa (C10) or a diet containing 10% cocoa plus 0.5% hesperidin (CH) for 6 weeks. In this period, animals were submitted to an intensive running training on a treadmill, involving three trainings per week and two exhaustion tests, or remained as a sedentary control group. Exercise performance was monitored throughout the study. At the end, samples were obtained 24h after performing a regular training (trained groups) and immediately after carrying out a final exhaustion test (exhausted groups) to assess the systemic immune status at different time points.

Results: None of the experimental diets modified the exercise performance. Both the C10 and the CH diets prevented the decrease in spleen Th proportion induced by the final exhaustion test. The C10 diet, but not the CH diet, avoided the exhaustion induced-plasma cortisol increase. In addition, the C10 diet induced a lower percentage of spleen Tαβ cells and a higher Tγδ cell proportion in the trained group, whereas those fed the CH diet showed an increased B cell percentage independently of the exercise condition. NK cytotoxicity was increased in rats fed the C10 diet, whereas the proportion of phagocytic monocytes decreased in both C10 and CH groups. Both diets differently modulated the serum immunoglobulin concentration and the in vitro immunoglobulin secretion by spleen lymphocytes.

Conclusion: Diets containing 10% cocoa, with or without 0.5% hesperidin, prevented the alterations in Th percentage induced by exhaustion and produced changes in the spleen lymphocyte proportions and functions beyond those effects of intensive exercise without affecting the exercise performance.

Dietary interventions with cocoa and hesperidin on the systemic immunity of intensively trained and exhausted rats

Patricia Ruiz-Iglesias [1,2], Malén Massot-Cladera [1,2], Francisco J. Pérez-Cano [1,2]* and Margarida Castell [1-3]*

[1]Secció de Fisiologia, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona (UB), 08028 Barcelona, Spain

[2]Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), UB, 08921 Santa Coloma de Gramenet, Spain

[3]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain

*Correspondence: franciscoperez@ub.edu (F.J.P.-C.); margaridacastell@ub.edu (M.C.);
Tel.: +34-93-402-45-05 (F.J.P.-C. & M.C.)

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Abstract

Purpose: To establish the influence of a cocoa-enriched diet and a cocoa- and hesperidin-enriched diet on the systemic immune alterations induced by intensive training and a final exhaustion test in rats.

Methods: Lewis rats were fed either a standard diet, a diet containing 10% cocoa (C10) or a diet containing 10% cocoa plus 0.5% hesperidin (CH) while they were submitted to an intensive running training on a treadmill. After 6 weeks, samples were obtained 24h after performing a regular training (T groups) and after carrying out a final exhaustion test (TE groups), to assess, among others, changes in the endocrine and humoral responses to exercise, as well as in the composition and function of spleen lymphocytes.

Results: The C10 diet attenuated the increase in plasma cortisol induced by exhaustion, while both the C10 and the CH diets prevented the alterations in the spleen Th cell proportion. The experimental diets also induced other changes, such as an increase in serum immunoglobulin concentration and an enhancement of spleen natural killer

cytotoxicity, which may be beneficial in situations with a weakened immunity. Most of the effects observed in the CH groups seem to be due to the cocoa content.

Conclusion: A dietary intervention with flavonoids enhances immune function, partially attenuating the alterations in systemic immunity induced by intensive training or exhausting exercise.

1. Introduction

Flavonoids are the most abundant polyphenols found in fruits and vegetables constituting around 75% of the total polyphenol intake in Europe, especially the flavanol and flavanone subclasses [1]. They have become a subject of increasing interest because of their numerous beneficial effects on human health [2, 3, 12, 4–11]. Their intake has been associated with lower risk of cardiovascular diseases [2], neurological disorders [3, 5], type 2 diabetes [6], obesity [7] and even cancer [8, 9]. In the athletic field, flavonoids and other polyphenols have been proposed as potential ergogenic aids [13], since they improve muscle function and mitochondrial biogenesis [14–17]. However, their effect on exercise performance varies among flavonoid subclasses and there is not yet enough clinical scientific evidence to draw a solid conclusion on their influence [13]. Nevertheless, because of their antioxidant [10], anti-inflammatory [11] and immunomodulatory [12] properties, their intake may be useful in the preventive management of oxidative stress [18], inflammation [19] and immune disruption [20], respectively, during intensive exercise.

It is well known that moderate activity exercise exerts several health benefits [21–23], such as enhancing immunity [21], up-regulating endogenous antioxidant enzymes [24] and reducing inflammation [22], thus decreasing the risk or improving the prognosis of several chronic diseases [23]. However, overly intense exercise may induce adverse effects on health, impairing immune function and leading to a higher risk of infections, especially those affecting the upper-respiratory tract (URTIs) and the gastrointestinal compartment [25, 26]. Intensive exercise induces changes in lymphoid compartments [27, 28]. In blood, exercise is followed by an intensity- and duration-dependent leukocytosis, mainly due to the mobilization of neutrophils and lymphocytes in response to a high release of catecholamines and glucocorticoids [29]. After exercise cessation, blood lymphocyte counts rapidly decrease until reaching a lymphopaenia that may last up to 6 h [29], which is due to a redistribution of T helper (Th) cells in both lymphoid and non-lymphoid organs [30], as well as to a higher apoptosis among highly

differentiated T cells [31]. Moreover, exercise can also modulate the function of immune cells, such as the cytotoxicity of Natural Killer (NK) cells, the phagocytic activity of blood phagocytes and the proliferation capacity of B and T cells, as well as their ability to secrete cytokines or produce immunoglobulins (Igs) [21, 27, 28, 32].

Flavonoids can modulate immune function and their intake is able to decrease the URTI incidence by 33% in a healthy population [33]. The most consumed flavonoid subclasses are flavanols and flavanones, mainly proanthocyanidins and hesperidin, respectively [1]. The main dietary sources of flavanols are cocoa products and green tea [1]. Cocoa flavonoid content comprises about 58% of proanthocyanidins, 37% of catechins, including (-)-epicatechin, (+)-catechin, (+)-gallocatechin and (-)-epigallocatechin, and, also, 4% of anthocyanidins [34]. On the other hand, flavanones are found in citrus fruits and hesperidin is the most abundant, especially the 2S-hesperidin isomer [35]. Preclinical and clinical studies have associated both cocoa and hesperidin consumption with reduced oxidative stress after exercise [36–41]. Moreover, cocoa has shown interesting effects on the mucosal immunity and the gut microbiota in a rat model of acute intensive exercise, which was partially mediated by its fibre content [42]. We have also demonstrated the ergogenic and immunomodulatory effects of hesperidin supplementation in a preclinical model of exhausting exercise [40, 43].

Considering this background, we hypothesize that a dietary intervention with cocoa and 2S-hesperidin could prevent the immune alterations induced by intensive training and exhausting exercise in rats, leading as well to a better exercise performance. Hence, we aimed to evaluate the effect of a cocoa-enriched diet and a cocoa- and hesperidin-enriched diet on the immune alterations present in rats submitted to a 6-week period of intensive running training and a final exhaustion test.

2. Materials and methods

2.1. Animals

Female Lewis rats (7-week-old; Janvier Labs, Saint-Berthevin, France) were maintained at the animal facility of the Faculty of Pharmacy and Food Science at the University of Barcelona (UB). The rats were housed in polycarbonate cages, 2–3 animals per cage, under controlled conditions of temperature and humidity in a 12 h light/12 h dark cycle. Female rats were chosen because they showed a better adaptation to the treadmill and exercise performance than male rats [32, 44], whereas the effects of exercise on

immunological variables was not influenced by rat gender [32]. Animal procedures were approved by the Ethical Committee for Animal Experimentation (CEEA) of the UB and the Catalonia Government (CEEA/UB ref. 517/18 and DAAM 10615, respectively).

2.2. Exercise and nutritional interventions

Before starting the nutritional intervention, to avoid biased distribution of animals, all rats were first familiarized with running on a treadmill (Exer3/6, Columbus, OH, USA), with an incline of 5 degrees, for one week by increasing both the running time and the treadmill speed. Afterwards, animals performed an exhaustion test (ET) in which, after 10 min running at 18 m/min, speed was increased 3 m/min every 2 min until rat exhaustion. This was established when rats touched the shock grid more than three times.

Rats were then homogeneously distributed into sedentary (SED) and runner (RUN) groups with the same average running capacity, and each group was also homogeneously distributed into three groups according to the diet: reference (REF), cocoa (C10) and cocoa plus hesperidin (CH). The REF groups (SED/REF and RUN/REF groups) received the standardized maintenance diet from the American Institute of Nutrition (AIN-93M, Envigo, Huntingdon, UK). The C10 groups (SED/C10 and RUN/C10 groups) were fed an isoenergetic diet containing 10% defatted cocoa (Idilia Foods S.L., Barcelona, Spain) providing a final proportion of 3.6 g/kg polyphenols, 6.0 g/kg soluble fibre and 54.0 g/kg insoluble fibre. The CH groups (SED/CH and RUN/CH) were fed the above described C10 diet containing an additional 0.5% of 2S-hesperidin (Cardiose®, HealthTech BioActives, Murcia, Spain), which is the predominant isomer found in citrus fruits [35].

Once the nutritional intervention began, the runner groups (i.e., RUN/REF, RUN/C10 and RUN/CH, n = 18 each one) were submitted to a 6-week intensive exercise programme, whereas the sedentary groups (i.e., SED/REF, SED/C10 and SED/CH, n = 12 each one) remained as non-exercised controls. In each week, rats carried out an ET every Monday and Friday, which consisted of running 15 min at 70% of the maximum speed average achieved in the previous Monday’s ET (the speed of the first Monday’s ET was 15 m/min), and from then on, the speed was progressively increased until exhaustion (3 m/min every 2 min). On Tuesday, Wednesday and Thursday, rats trained for 25, 30 and 40 min, respectively, at 70% of the maximum speed achieved in the previous Monday’s ET.

At the end of the 6-week training programme, each RUN/REF, RUN/C10 and RUN/CH group was distributed into two subgroups with the same running capacity, which allowed the immune function assessment at different time points: trained groups (T/REF, T/C10 and T/CH, n = 9 each one), whose samples were obtained 24 h after a regular training session, and trained and exhausted (TE/REF, TE/C10 and TE/CH groups, n = 9 each one), whose samples were obtained after performing an additional final exhaustion test, where the initial speed was maintained for 30 min instead of the 15 min applied in the weekly ETs.

Throughout the study, food and water were provided ad libitum and their consumption was monitored, as well as the rats' body weight and exercise performance.

2.3. Sample collection

At the end of the study, animals were anaesthetized (ketamine, 90 mg/kg, Merial Laboratories S.A., Barcelona, Spain; xylazine, 10 mg/kg, Bayer A.G., Leverkusen, Germany) and exsanguinated. Blood was immediately analysed using an automated haematologic analyser (Spincell, MonLab Laboratories, Barcelona). Another blood sample was used for assessing the phagocytic activity of monocytes and granulocytes. Other blood samples were used to obtain plasma and serum, which were maintained at -80 °C or -20 °C until hormone and Ig quantification, respectively. Heart, liver, thymus, spleen and gastrocnemius were collected and weighed. Lymphocytes from spleen were isolated and used for characterizing the lymphocyte composition and function.

2.4. Plasma cortisol and noradrenaline concentration

Plasma cortisol concentration was determined with the DetectX® Cortisol competitive enzyme-linked immunosorbent assay (ELISA, Arbor Assays, MI, USA) following the manufacturer's protocol. Plasma noradrenaline concentration was quantified using the Noradrenaline/Norepinephrine (NA/NE) competitive ELISA Kit (Elabscience, TX, USA) following the manufacturer's protocol. In both cases, absorbance was measured on a microplate photometer (Labsystems Multiskan, Helsinki, Finland) and data were interpolated by Ascent v.2.6 software (Thermo Fisher Scientific, Barcelona, Spain) according to the respective standard curves.

2.5. Spleen lymphocyte isolation and phenotypic analysis

Spleen lymphocytes were isolated in aseptic conditions by smashing the tissue in a sterile mesh cell strainer (40 µm, Thermo Fisher Scientific) as previously described [27]. After erythrocyte lysis, splenocyte numbers and viability were determined by a Countess Automated Cell Counter (Invitrogen, Thermo Fisher Scientific). Afterwards, the proportion of the different lymphocyte subsets was assessed by flow cytometry using mouse anti-rat CD161b, CD45RA, CD8 α, CD4, TCR $\alpha\ \beta$ or TCR $\gamma\ \delta$ monoclonal antibodies (mAb) (BD Biosciences, CA, USA) conjugated either to fluorescein isothiocyanate (FITC), phycoerythrin, peridinin-chlorophyll-a protein, allophycocyanin or brilliant-violet 421, as described previously [27]. A negative control staining without mAb and a staining control for each mAb were included. Data were acquired with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the Flow Cytometry Unit (FCU) of the Scientific and Technological Centers of the UB (CCiTUB) and analysed with FlowJo v.10 software (Tree Star, Inc., Ashland, OR, USA). The percentage of positive cells in the lymphocyte population selected was established according to forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) or in a particular lymphocyte population.

2.6. Spleen lymphocyte stimulation and proliferation

Spleen lymphocytes (5 x 105 cells) were incubated in quadruplicate in 96-well plates (TPP, Sigma-Aldrich, Madrid, Spain) and stimulated or not with Concanavalin A (ConA, 5 µg/mL, Sigma-Aldrich). After 48 h, supernatants were collected and stored at -80 °C until cytokine and Ig quantifications, while T cell proliferation ability was assessed using a BrdU Cell Proliferation Assay Kit (Roche, Madrid, Spain), according to the manufacturer's instructions. The proliferation rate was calculated by dividing the absorbance of ConA stimulated cells by that of non-stimulated cells.

2.7. Cytokine and immunoglobulin quantification

The concentration of IgG1, IgG2a, IgG2b, IgG2c, IgM and IgA in plasma and non-stimulated splenocyte supernatants and that of interleukin (IL) 2, IL-10, interferon (IFN) γ, granulocyte colony-stimulating factor (G-CSF), granulocyte and macrophage colony-stimulating factor GM-CSF, IL 4, IL-6, IL-1 α, IL-1 β and tumour necrosis factor (TNF) α in ConA-stimulated splenocyte supernatants were quantified at the end of the study

using ProcartaPlexTM Multiplex immunoassay (Affymetrix, eBioscience, San Diego, USA), according to the manufacturer's protocol. Data were acquired by MAGPIX Cytometer (Affymetrix) in the FCU of the CCiT-UB and analysed by ProcartaPlex Analyst v1.0 software (Affymetrix). The lower limits of quantification (LLOQ) were: 1.70 ng/mL for IgG1, 1.73 ng/mL for IgG2a, 2.67 ng/mL for IgG2b, 3.67 ng/mL for IgG2c, 0.197 ng/mL for IgM, 0.584 ng/mL for IgA, 1.82 pg/mL for IL-2, 6.01 pg/mL for IL-10, 3.34 pg/mL for IFN-γ, 4.91 pg/mL for G-CSF, 4.81 pg/mL for GM-CSF, 0.62 pg/mL for IL- 4, 2.19 pg/mL for IL-6, 10 pg/mL for IL-1α, 13 pg/mL for IL-1β and 2.88 pg/mL TNFα. Total IgG was calculated as the addition of the four isotypes and the Th1/Th2 ratio was calculated by dividing the concentration of the Th1-associated isotypes (IgG2b and IgG2c) by that of the Th2-, associated isotypes (IgG1 and IgG2a). In splenocyte supernatants, IgG2a, G-CSF, GM-CSF, IL-4, IL-6, IL-1α, IL-1β and TNFα levels were below the LLOQ.

2.8. Phagocytic activity

The proportion and phagocytic activity of monocytes and granulocytes were quantified in blood samples using the PhagotestTM kit (Glycotope, Biotechnology GmbH, Heidelberg, Germany), following the manufacturer's instructions as previously reported [32]. Data were acquired using Gallios™ Cytometer in the FCU of the CCiTUB and the analysis of the results was carried out with FlowJo v.10 software. Monocyte and granulocyte subsets were selected according to their FSC/SSC. The proportion of FITC positive cells in each gate was considered as the percentage of monocytes and granulocytes with phagocytic ability present in the sample, whereas the mean fluorescence intensity (MFI) indicated their corresponding phagocytic activity. Changes in both blood cells' phagocytic proportions and activities are represented considering the SED/REF group mean value as 1, therefore, all values are expressed as a fold change of the mean value with respect to the SED/REF group.

2.9. Natural Killer (NK) cell cytotoxic activity

The cytotoxic activity of spleen NK cells was determined by the NKTESTTM kit (Glycotope), according to the manufacturer's instructions as previously described [32]. Data were acquired using Gallios™ Cytometer (FCU in the CCiTUB) and the analysis of the results was carried out with FlowJo v.10 software. The individual cytotoxic activity

was calculated according to the total NK activity and the percentage of NK cells of each sample.

2.10. Statistical analysis

Statistical analysis of the data was performed using IBM Social Sciences Software Program (SPSS, version 26.0, Chicago, IL, USA) and Rstudio v4.04 (Rstudio, Inc.) with R version 3.6.1 (R Core Team 2021, R Foundation for Statistical Computing, Vienna, Austria). The normality and homoscedasticity of the data were tested by Shapiro–Wilk's and Levene's test, respectively. Once these conditions were confirmed, a two-way ANOVA test was applied and, if significant differences were detected, Tukey's post hoc test was carried out. Otherwise, non-parametric Aligned Rank Transform for non-parametric factorial ANOVA (ART-ANOVA) followed by emmeans post hoc (Tukey-adjusted p value) were applied, using the ARTool [45, 46] and emmeans [47] packages, respectively, for Rstudio. To compare variables during the study (e.g. body weight, daily chow and water intake, and changes in distance run in the exhaustion tests), a repeated-measures ANOVA was applied. Significant differences were considered when $p \leq 0.05$. When significant differences were detected, the p values obtained in the two-way ANOVA or the ART-ANOVA for the variables diet (D), exercise (E) and the interaction between them (DxE) were written in the legend box. Changes due to the dietary condition were represented with symbols in the legend box. Changes due to the exercise condition were represented in the figure using different letters above the bars. When the DxE interaction was significant, changes between groups were represented with symbols above the respective bars.

3. Results

3.1. Body weight, chow intake and training performance

Body weight (BW), chow and water intake were monitored three times per week throughout the 6 weeks of the study (**Fig. 1**). The initial BW was similar among all the groups, but throughout the study, the animals fed C10 and CH diets, both SED and RUN groups, had a lower BW gain (**Fig. 1A**). This effect was not associated with a lower chow intake; and on the contrary, the intake of both experimental diets was increased (**Fig. 1B**). The water intake was also higher in the C10 and CH groups, which was particularly visible during the first weeks of the nutritional intervention (**Fig. 1C**).

With regard to exercise performance, neither the C10 nor the CH diets modified the distance run in the weekly ETs (Fig. 1D). Similarly, there were no differences in the performance of the final ET, which was slightly different to the weekly ones because the initial speed was maintained for 30 min instead of 15 min. In the final ET, the RUN/REF group ran 1434.7 ± 62.48 m, the RUN/C10 group 1593.9 ± 81.00 m and the RUN/CH group 1439.9 ± 85.27 m.

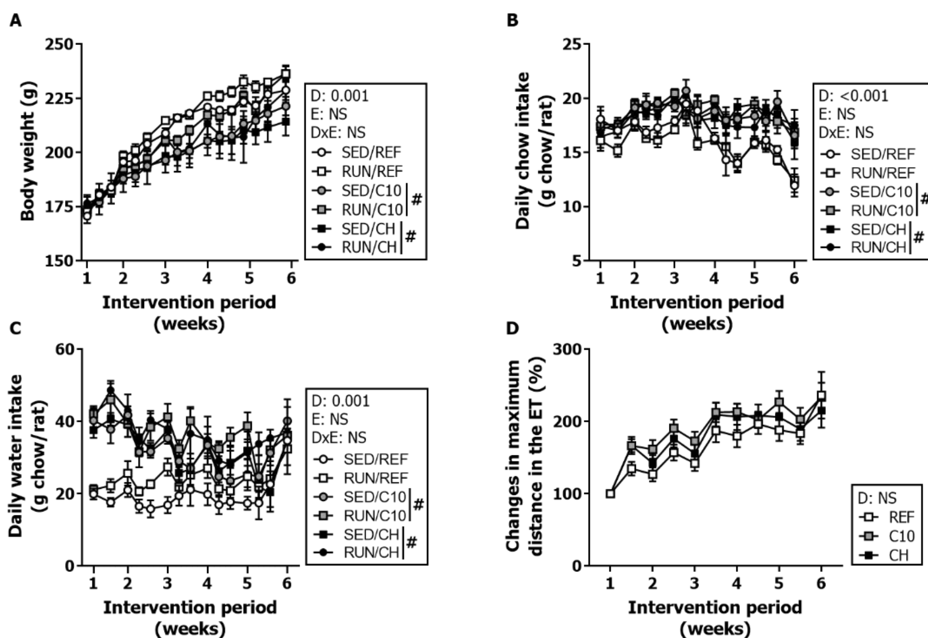

Fig. 1. Body weight (A), chow intake (B) and water intake (C) throughout the 6-weeks of training and nutritional intervention, and changes in the maximum distance run in the exhaustion tests (ETs) performed throughout the study with respect to the first ET in the runner rats (D). Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); runner rats (RUN); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (n = 6–18). Statistical differences: #p<0.05 vs REF diet; NS, no statistically significant differences detected.

On the other hand, the C10 diet decreased plasma cortisol in the three exercise conditions (SED, T and TE), preventing the increase induced by the final exhaustion test (Fig. 2A). Neither the exercise undergone nor the experimental diets induced significant changes in plasma NA, although the C10 diet tended to increase it independently of the exercise condition (p= 0.081) (Fig. 2B).

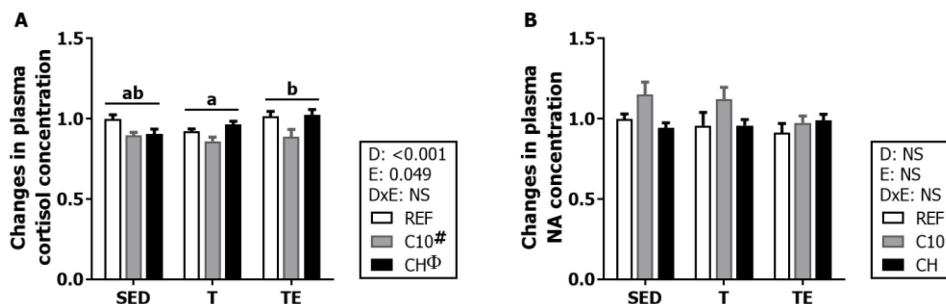

Fig. 2. Changes in cortisol (**A**) and noradrenaline (NA) (**B**) concentration in plasma at the end of the study compared to the SED/REF group. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (n = 9–12). Statistical differences: $p<0.05$ between values not sharing common letters; # $p<0.05$ vs REF diet; Φ $p<0.05$ vs C10 diet; NS, no statistically significant differences detected.

3.2. Organ weight

At the end of the study, the relative weight of the heart, liver, thymus, spleen and gastrocnemius muscle was determined (**Fig. 3**). All exercised rats (T and TE groups) had a higher gastrocnemius weight independently of the diet. The relative weight of the heart and the liver was not modified by any exercise condition or the experimental diets.

With regard to immune tissues, in comparison with the respective T groups, the final exhaustion test induced a decrease in the relative weight of the spleen for all animals. Moreover, C10 and CH diets also reduced the spleen relative weight. On the other hand, in REF animals, the final exhaustion test decreased thymus weight with respect to the SED animals. Additionally, the intake of the experimental diets reduced thymus weight in almost all conditions.

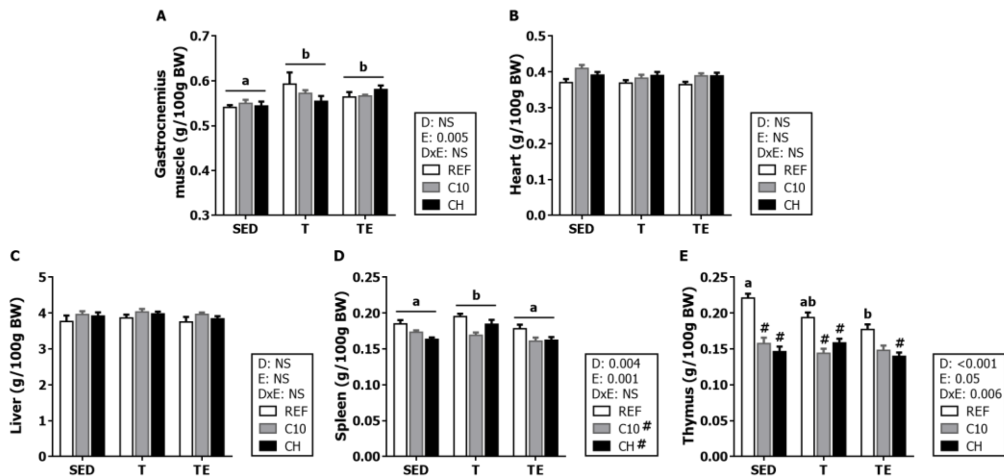

Fig. 3. Organ relative weights at the end of the study. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (*n* = 9–12). Statistical differences: *p*<0.05 between values not sharing common letters; # *p*<0.05 vs REF diet; NS, no statistically significant differences detected.

3.3. Haemograme

Blood leukocyte counts were higher after the 6-week exercise training (T groups) but not immediately after exhaustion (TE groups) (Fig. 4A). With regard to differential leukocyte proportions (Fig. 4B-D), exercise did not modify the percentage of lymphocytes, granulocytes or monocytes.

On the other hand, the consumption of the C10 diet increased the number of circulating leukocytes. This increase seems to be due to a higher proportion of granulocytes, since the lymphocyte and monocyte proportions decreased or remained unchanged, after the intake of the C10 diet. The effects on lymphocyte and granulocyte proportions were also observed after the intervention with the CH diet.

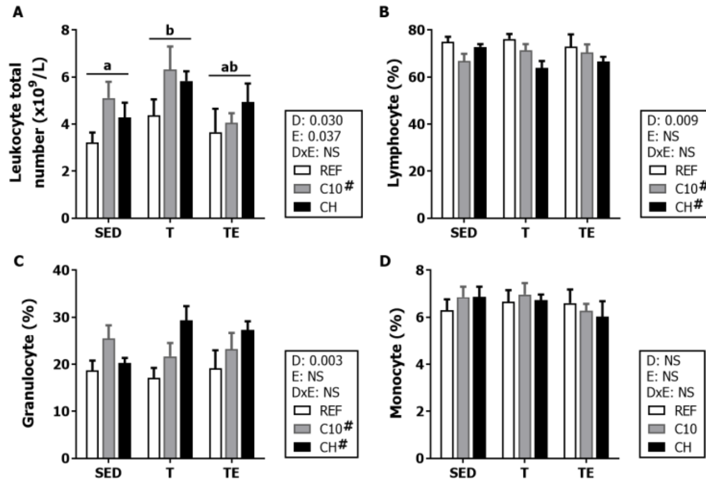

Fig. 4. Blood counts of leukocytes (A) and percentage of lymphocytes (B), granulocytes (C) and monocytes (D) at the end of the study. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (*n* = 6–8). Statistical differences: $p<0.05$ between values not sharing common letters; # $p<0.05$ vs REF diet; NS, no statistically significant differences detected.

With regard to red blood cell variables, the erythrocyte counts and the haemoglobin concentration decreased immediately after the final exhaustion (TE groups vs SED groups) in all dietary conditions (Fig. 5). The experimental diets did not significantly modify these variables, although the CH diet tended to raise the haemoglobin levels (p= 0.07). Neither exercise nor the diets significantly modified the haematocrit, although the intake of the CH diet tended to increase it (p= 0.07).

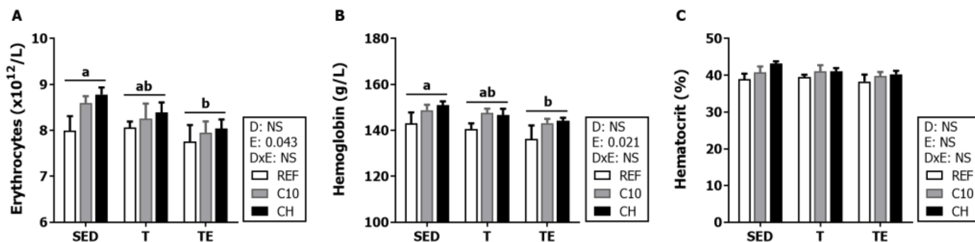

Fig. 5. Blood counts of erythrocytes (A); haemoglobin concentration (B); and hematocrit (C) at the end of the study. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (*n* = 6–8). Statistical differences: $p<0.05$ between values not sharing common letters; NS, no statistically significant differences detected.

3.4. Serum immunoglobulins

Neither the exercise training nor the final exhaustion test modified the concentration of IgM and IgA in serum at the end of the study (**Fig. 6A-B**). However, the C10 and the CH diets increased the serum IgA concentration, and the C10 diet also raised that of IgM.

The serum IgG concentration was higher after the final exhaustion (TE groups vs SED groups) (**Fig. 6C**). Moreover, animals fed the C10 diet, independently of the exercise condition, showed threefold higher serum IgG content. With regard to the different IgG isotypes, exercise did not modify their proportion but both the C10 and the CH diets decreased the proportion of IgG1 and IgG2a (**Fig. 6D-E**). When considering IgG2c (the most abundant), the CH diet significantly increased it immediately after the final exhaustion test (**Fig. 6G**). Considering IgG2b and IgG2c as representative of Th1 response, and IgG1 and IgG2a representative of the Th2 response, the ratio Th1/Th2 (**Fig. 6H**) showed about a two- to threefold increase as a result of the intake of both experimental diets.

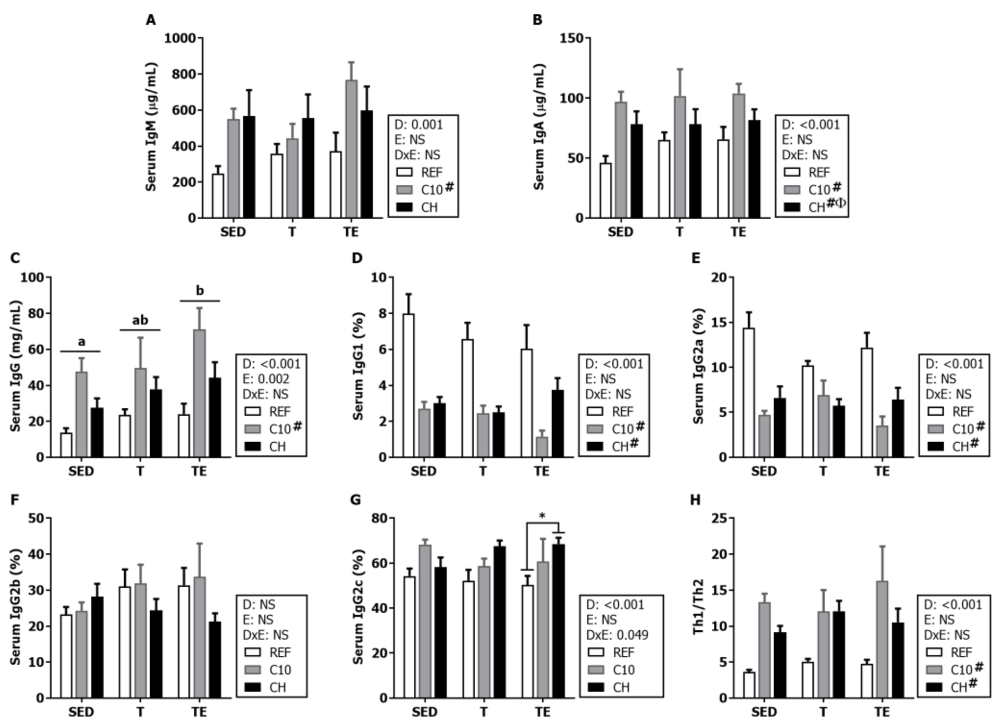

Fig. 6. Immunoglobulins (Ig) concentration in serum at the end of the study. The IgG subclasses (**D**–**G**) were calculated as the percentage of each particular isotype concentration with respect

to the total IgG concentration. The Th1/Th2 ratio (**H**) was calculated dividing IgG2b+IgG2c and IgG1+IgG2a concentrations. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (n = 9–12). Statistical differences: $p<0.05$ between values not sharing common letters; * $p<0.05$; # $p<0.05$ vs REF diet; Φ $p<0.05$ vs C10 diet; NS, no statistically significant differences detected.

3.5 Phagocytic activity

The proportion of phagocytic monocytes and granulocytes, and their phagocytic activity were quantified (**Fig. 7**). The SED/REF animals had a 63.88 ± 4.35 % of phagocytic monocytes (with respect to total monocytes) and a 93.42 ± 1.24 % of phagocytic granulocytes (with respect to total granulocytes).

The percentage of phagocytic monocytes was influenced both by intensive training and the experimental diets. The 6-week training induced a decrease in the monocyte proportion (T groups vs SED groups). Additionally, final exhaustion decreased their phagocytic activity (TE groups vs T groups). Both the C10 and the CH diets induced a decrease in the phagocytic monocyte proportion without significantly affecting their functionality.

No changes in the proportion of phagocytic granulocytes were observed due to exercise or the experimental diets. However, the granulocyte phagocytic activity was increased by training and the final exhaustion test (T and TE groups vs SED groups).

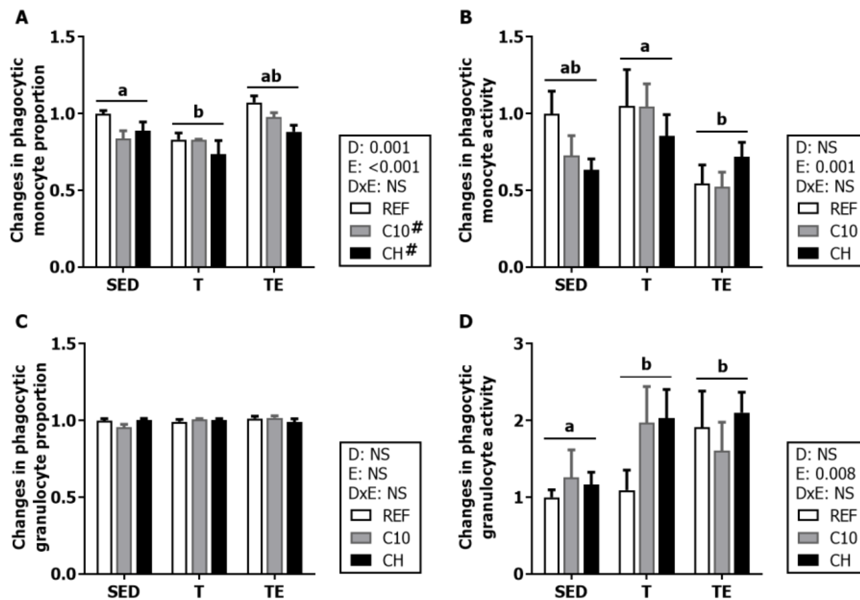

Fig. 7. Changes in blood phagocytic monocyte proportion (**A**); blood monocyte phagocytic activity (**B**); blood phagocytic granulocyte proportion (**C**); and blood granulocyte phagocytic activity (**D**) compared to the SED/REF group. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (n = 6–8). Statistical differences: p<0.05 between values not sharing common letters; # p<0.05 vs REF diet; NS, no statistically significant differences detected.

3.6 Spleen lymphocyte composition and function

The 6-week exercise training did not modify the major lymphocyte populations' Tαβ cells, B cells, NK cells or Tγδ cells in REF animals (**Fig. 8**). However, the C10 diet decreased Tαβ cell proportion in the T group while the CH diet increased that of B cells in all exercise conditions. With regard to the percentage of Tγδ cells, trained rats fed C10 diet showed a higher percentage of Tγδ cells than their REF counterpart. Neither exercise nor the experimental diets modified the proportion of NK cells.

With regard to the proportion of Th and Tc cells in Tαβ cells, Th cell percentage decreased after exhaustion with respect to trained conditions (the TE groups vs T groups) but both the C10 and the CH groups showed higher Th cell proportions than the REF groups. The experimental diets reciprocally decreased the proportion of Tc cells, without modifying that of NKT cells.

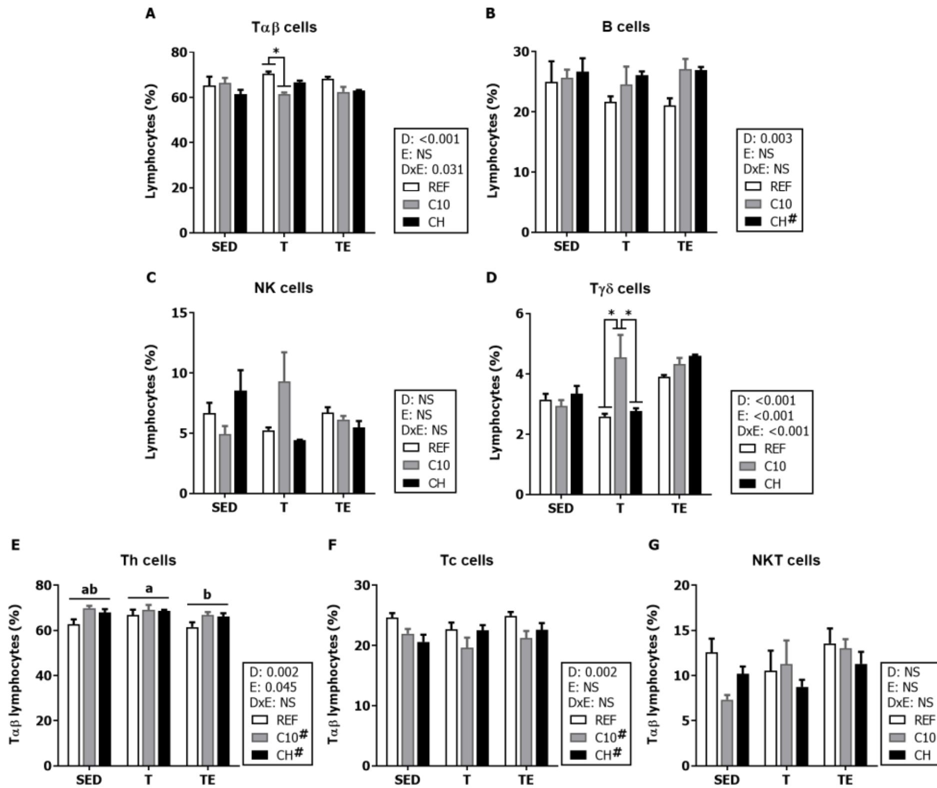

Fig. 8. Percentage of spleen lymphocytes: T (TCRαβ+) (**A**); B (CD45RA+) (**B**); natural killer (NK) (CD161b+ TCRαβ-) (**C**); Tγδ (TCRγδ+) (**D**); Th (CD4+CD161b- in TCRαβ+) (**E**); Tc (CD8+CD161b- in TCRαβ+) (**F**); and NKT (CD161b+ in TCRαβ+) (**G**) cells. Diet (D); exercise (E); diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (n = 6–8). Statistical differences: p<0.05 between values not sharing common letters; * p<0.05; # p<0.05 vs REF diet; NS, no statistically significant differences detected.

The impact of exercise and the nutritional interventions on the function of spleen lymphocytes was also studied (**Fig. 9–10**). At the end of the study, the splenocytes proliferation ability was similar among all the groups (**Fig. 9A**), while the cytotoxicity of NK cells was higher in those fed C10 diet (**Fig. 9B**).

The splenocyte cytokine secretion capacity after in vitro stimulation was also assessed (**Fig. 9C–E**). In SED/REF animals, splenocytes secreted 625.7 ± 316.31 pg/mL of IL-10, 1112.9 ± 354.18 pg/mL of IL-2, and 9337.7 ± 1564.15 pg/mL of IFN-γ. The cells collected after the final exhaustion test (TE groups) produced lower levels of IL-10 and IL-2 than

trained groups. None of the experimental diets modified the secretion of the studied cytokines.

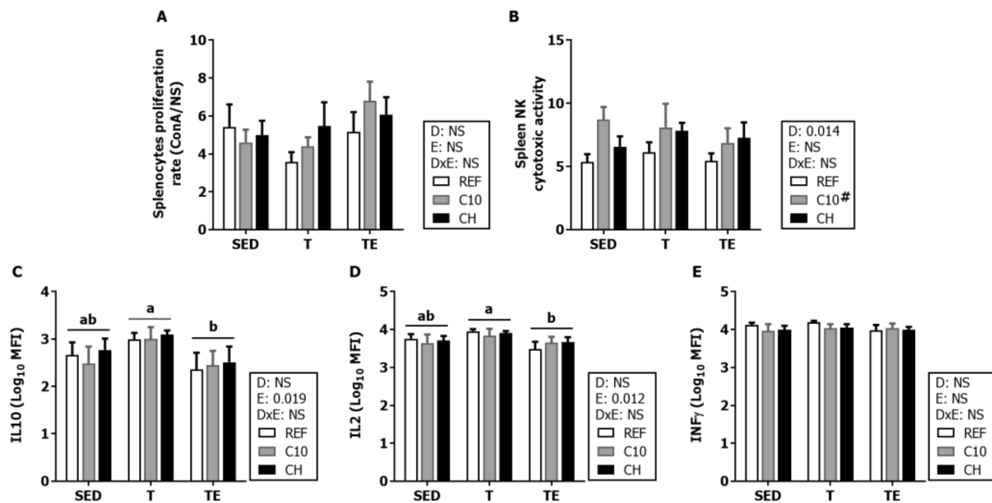

Fig. 9. Spleen lymphocyte functionality: proliferation response (**A**), NK cytotoxicity (number of dead target cells per 100 effector cells) (**B**), and cytokine release under concanavalin A (ConA) stimulation (**C**–**E**). Diet (D); exercise (E), diet x exercise interaction (DxE); sedentary rats (SED); trained rats (T); trained and exhausted rats (TE); reference diet (REF); cocoa diet (C10); cocoa and hesperidin diet (CH). Data are expressed as mean ± standard error of the mean (SEM) (n = 6–8). Statistical differences: p<0.05 between values not sharing common letters; # p<0.05 vs REF diet; NS, no statistically significant differences detected.

Finally, the in vitro ability for Ig production was assessed in supernatants from non-stimulated spleen lymphocytes (**Fig. 10**). Neither training nor the final ET modified the secretion of the Ig isotypes evaluated. Both the C10 and the CH diets decreased the proportion of IgG2b (**Fig. 10E**). The CH diet increased the concentration of IgM and lowered that of IgA, while the proportion of IgG2c in the T group also increased.

4. Discussion

We have evaluated the effect of a chronic and intensive training (T group) and an additional exhaustion test (TE group) on some biomarkers of the immune system, and we have also assessed the influence of a 10%-cocoa diet (C10 diet) and a cocoa diet containing hesperidin (CH diet) in these biomarkers. We observed some changes

induced by training, such as a higher granulocyte phagocytic activity, and others produced by the additional exhaustion test, such as a higher Th percentage in the spleen and a decreased blood monocyte phagocytic activity. When considering the protective effects of the experimental diets (C10 and CH diets) on the changes in the immune biomarkers, we observed other effects beyond those induced by exercise: both experimental diets prevented the Th decrease induced by the exhaustion test and decreased the proportion of phagocytic monocytes, and the C10 diet induced a higher cytotoxic activity. The serum immunoglobulin concentration and the in vitro immunoglobulin secretion were changed by the experimental diets, inducing higher serum concentrations of IgM, IgA and IgG, particularly of those isotypes linked to Th1 activity.

Firstly, considering the exercise performance, we did not observe an improvement after 6 weeks of nutritional interventions with cocoa or cocoa plus hesperidin. This result agrees with most of the preclinical and clinical studies involving cocoa products, such as cocoa powder [38, 42, 48], cocoa flavanol capsules [37] or chocolate [36, 49, 50]. With regard to hesperidin, both preclinical [40, 41, 43] and clinical studies [35, 39, 51, 52] have reported its ergogenic effects. The dose of the flavanone used in such preclinical studies was about 600-700 mg/kg per week [40, 43]. In the current study, in terms of the average daily rat food intake, the diet we used meant a consumption of 3150 mg/kg BW of hesperidin per week, which was much higher than that provided in the studies that supplemented by oral gavage [40, 41, 43]. The lack of an ergogenic effect of the CH diet may be due to some interaction between hesperidin and the cocoa components, as well as the fact that rats took the hesperidin in the food throughout the day, meaning a slower intake rate. Further studies should elucidate the importance of the method of administration of hesperidin or other flavonoids alone to achieve an ergogenic effect.

The spleen is the largest secondary lymphoid organ in the body and plays an essential role in maintaining immune homeostasis. Six weeks of intensive training induced a higher relative spleen weight with no changes in the main spleen lymphocyte subsets. After the additional exhaustion test, spleen relative weight decreased, but there were no changes in the main lymphocyte proportions. These results agree with a similar study, where intensive training or exhaustion did not alter spleen lymphocyte proportions [43]. However, in the current study, after exhaustion, the proportion of the spleen Th cell subset decreased, which was prevented by the intake of the C10 and CH diets. This effect could probably be attributed to cocoa, since cocoa intake in non-exercised rats induced a similar effect in a previous study [53]. On the other hand,

trained rats fed C10 diet had a lower proportion of Tαβ cells and a higher one of Tγδ cells, which is in line with results in the mesenteric lymph nodes of non-exercised rats [54]. In addition, the CH diet induced a higher proportion of B cells, which may be attributed to both cocoa and hesperidin, since the C10 groups also tended to have higher levels of B cells, which is in line with previous studies [55]. With regard to spleen NK cells, although neither exercise nor the diets modified their proportion, the C10 diet enhanced their cytotoxic activity in all exercise conditions. The effect of cocoa on NK activity could be related to its antioxidant effect, since oxidative stress seems to reduce the expression of the activating receptor NKG2D in NK cells [56].

After 6 weeks of intensive exercise, trained rats had a higher number of blood leukocytes and, after exhaustion, there was a decrease in the erythrocyte counts and haemoglobin concentration, which could be due to the haemodilution induced by plasma volume expansion in the recovery period [57]. None of the experimental diets prevented the changes in red and white blood cells. Indeed, animals fed the C10 diet had even higher leukocyte counts than those fed REF diet, which was due to a higher granulocyte proportion, in agreement with the results obtained in a clinical study about acute exercise [58]. On the other hand, the final exhaustion restored the increased leukocyte counts achieved in trained rats. It is well documented that a single bout of intensive exercise is followed by an immediate increase in blood leukocytes [29] which has been attributed to noradrenaline release [29]. Here, exhausted rats did not show increased plasma noradrenaline concentration, although they did have higher plasma cortisol levels. This must be due to the fact that the sympathetic nervous system activation during exercise precedes that of the hypothalamic–pituitary–adrenal axis and cortisol release [29]. In fact, some studies report the decrease of catecholamine increased levels after 10 and 60 min of intensive exercise [59, 60]. These studies denote the importance of the time of sampling. Here, although we aimed to assess the changes immediately after exercise cessation, technical limitations meant that blood collection was actually performed 20-30 min after extenuation, which may be enough to normalize the number of circulating leukocytes and the concentration of plasma noradrenaline. On the other hand, cocoa diet attenuated the cortisol increase, which is in line with previous studies [38, 61], but this did not affect the leukocyte counts.

Exercise and the experimental diets affected the blood phagocytic activity. Training and exhaustion increased granulocyte phagocytic activity, although exhaustion decreased that of blood monocytes. The higher phagocytic granulocyte activity agrees with a study performed in mice, in which an increased bronchoalveolar macrophages phagocytic capacity was found after running to exhaustion on a treadmill [62]. This must be due to

the release of glucocorticoids and catecholamines induced by intensive exercise [63]. None of the experimental diets influenced the phagocytic activity, although they did lower the proportion of monocytes with phagocytic capacity. These effects may be attributed to the cocoa consumption because it has been reported that cocoa reduces the expression of scavenger receptors and adhesion molecules such as the integrin VLA-4 and L-selectin, as well as that of CD40 and CD36 [64].

With regard to serum immunoglobulins, the final exhaustion test increased IgG concentration, which is consistent with both preclinical [27] and clinical studies [65–67]. This could be explained by the longer IgG half-life [65] observed after the chronic practice of moderate intensity exercise. The consumption of the C10 diet increased serum IgG, IgM and IgA concentrations, which was quite surprising because in previous preclinical studies no changes [68] or even decreased levels [69] were found in these Igs. The controversy among these results may be caused by the use of different rat strains, ages or sexes, as well as different cocoa batches. With regard to the different IgG isotypes, exercise did not modify their concentrations, although the intake of the experimental diets decreased the levels of those isotypes linked to the Th2 antibody response in rats, which agrees with previous studies reporting the antiallergic potential of cocoa [70]. Moreover, the CH diet in exhausted animals increased the serum IgG2c concentration, which was also increased in the splenocyte supernatants from the CH-fed trained rats. A previous study using rats of a similar age also found higher serum levels of IgG2c after a dietary intervention with cocoa [71], although another study using younger animals found the opposite effect [53], denoting the importance of age when assessing changes on immune function. On the other hand, both the C10 and the CH diets induced higher serum levels of IgA, which may enhance protection against potential viral infections, such as URTIs, whose risk is increased in athletes [25, 26]. Further studies may clarify the real impact of these changes, assessing their potential protective effect after the induction of a viral infectious process.

With regard to the in vitro production of immunoglobulins, there was no effect of exercise, but the CH diet increased the IgM secretion, which could be associated with the higher spleen proportion of B cells observed in these animals. Neither exercise nor the diets modified the IgG in splenocyte supernatants, with the exception of a decrease in the IgG2b isotype production induced by the experimental diets.

Overall, our results evidence some immune changes in intensively trained Lewis rats, such as a higher granulocyte phagocytic activity. In addition, when a final exhaustion test was performed, a lower Th percentage and IL-2 and IL-10 secretion in spleen were found, whereas high granulocyte phagocytic activity was maintained. Diets containing

10% cocoa, with or without 0.5% hesperidin, prevented the alterations in Th percentage induced by exhaustion and produced changes in the spleen lymphocyte proportions and functions beyond those effects of exercise. Nevertheless, the experimental diets did not improve exercise performance. Further research may evaluate the influence of these and other flavonoid-enriched diets in a more intensive exercised protocol, which may exacerbate the observed immune alterations, as well as assess their potential protective effect in the induction of an infectious process after exercise.

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Author contributions: M.C. and F.J.P.–C. conceived and designed the experiments; P.R.-I. and M.M.-C. performed the experiments, P.R.-I. analysed the data and wrote the paper, and M.M.-C., M.C. and F.J.P.–C. reviewed the manuscript. All authors have read, reviewed and approved the final version of the manuscript.

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Compliance with ethical standards: The manuscript does not contain clinical studies or patient data.

Conflict of interest: The authors declare that they have no conflict of interest.

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DISCUSSION

It is It is well known that the regular practice of moderate intensity exercise offers many health benefits. Among others, moderate exercise can boost the immune system, increasing the hosts' immune surveillance. However, during periods of intensive training, an increased incidence of respiratory and gastrointestinal infections has been observed in athletes, which may be the result of mucosal immune dysfunction. These harmful effects of intensive exercise have gained increasing attention from researchers lately, mainly due to the growing participation of the general population in sport competitions. Nevertheless, the isolated role of exercise on the higher risk of infection and immune function is still unclear. In human studies, many uncontrolled factors can influence immune function, such as the physiological stress associated with competitions, changes on the dietary and sleep patterns and travel. In addition, attending a mass participation event per se increases the exposition to pathogens, which makes it difficult to attribute the higher incidence of infections observed in athletes to the immune alterations induced by exhausting exercise. For this reason, an optimal animal model of intensive exercise training can be useful. Previous studies from our research group have demonstrated that intensive training and exhausting exercise influence the innate and the systemic adaptive immune system in rats (55,160). Nevertheless, none of these studies looked at changes on the mucosal compartment.

In the current thesis, the first objective aimed to evaluate the influence of exercise on the mucosal immune system and the gut microbiota. We firstly focused on the gut microbiota (**Article 1**) and then, in a more intense exercise model, on the intestinal barrier function and the changes in the own mucosal immune system (**Article 2**). In addition, this model was used in subsequent studies to further characterize the effects of intensive training alone or followed by exhausting exercise on immune system (**Articles 5 and 6**).

Firstly, we established the changes in caecal microbiota composition in a model of intensive training in male and female Wistar rats that performed two 30-min running sessions per day (6 h between sessions) for 15 days followed by a final exhaustion test (ET) (**Article 1**). This model was based on previous studies (348,349). We chosed to use Wistar rats (4-week-old) based on the results from previous studies using similar exercise models (55,350–353). This preliminary phylogenetic study, carried out by 16S rRNA sequencing technique, allowed us to conclude that male and female rats showed a different microbiota pattern. On the other hand, no training-associated changes were observed on microbiota diversity and richness indexes, although the running training produced an increase in the proportion of *Paraeggerthella* genus (from Actinobacteria phylum) in female rats and a decrease in that of *Bifidobacterium* spp. in male rats,

whose basal levels were higher than in female rats. Qualitative changes in caecal microbiota composition were also observed after intensive training and exhausting exercise. Some families were just present in sedentary rats while others colonized the caecal microbiota after exercise, among them, the family *Staphylococcaceae* was exclusively present in male runner rats and the family *Coriobacteriaceae*, which in mice increases in response to stress (354), in female runner rats. Estruel-Amades *et al.* (55,160) previously characterized the changes on immune system induced by this training programme in these rats. They found a lower proportion of Tαβ cells (in particular Th cell subset) in the thymus, which may suggest a mobilization of mature thymocytes into the blood. The other assessed biomarkers of innate and adaptive immune function were barely modified by this exercise model but, from the results obtained, it was clear that female rats had a higher ability to run than males. In summary, from the **Article 1** and parallel studies carried out in these animals (55,160), it can be concluded that, in Wistar rats, sexual dimorphism influences the runner ability and caecal microbiota composition, being female rats better runners than male rats. In addition, caecal microbiota composition is modified by intensive training at both a quantitative and qualitative level.

Following with the same objective of the thesis, we next aimed to establish the influence of exercise on the mucosal immune system. Given that the exercise model previously detailed did not induce substantial changes in the systemic immunity (55,160), to achieve this next goal, we used a longer and more intense rat model of training and exhausting exercise, already applied previously by Estruel-Amades *et al.* (55,160) and based on that described by Batatinha *et al.* (355). Based on our own experience and results reporting that female rats were better than male rats in terms of adaptability to the treadmill (55,350–353), we decided to use female rats for the following experiments. The training programme applied then lasted for 5 weeks, during which the rats performed, weekly, five exercise sessions: three regular trainings and two ETs. Every Monday and Friday, the rats exercised to exhaustion at increasing speeds, while on Tuesday, Wednesday and Thursday they ran for 20, 25, and 30 min, respectively, at 60% of the maximum speed average achieved on the previous Monday ET (between 34-43 m/min). After 5 weeks, the rats performed an additional ET. Samples were collected either before (T group), immediately after (TE group) or 24 h after (TE24 group) performing the ET to assess the influence of both the chronic intensive training and the final session of exhausting exercise (the acute effects and those that remain even during the recovery period). Using this model, Estruel-Amades *et al.* previously characterized the alterations in the innate (55) and systemic adaptive (160) immune system. They concluded that this rat model of intensive training for 5 weeks reduced

the phagocytic activity and the proliferative capacity of spleen lymphocytes, while increased the reactive oxygen species (ROS) release by peritoneal macrophages, the cytotoxicity of NK cells and the systemic production of immunoglobulin (Ig) G, among other changes. On the other hand, the intensively trained rats that also undergone a final ET had leukocytosis and a higher monocyte phagocytic activity than both sedentary and trained rats that were not submitted to the final exhaustion. In the current thesis, we aimed to characterize the alterations in the mucosal immune system in this exercise model (**Article 2**). On the one hand, we found that the intensive training decreased the concentration of IgA in salivary glands, in line with most previous studies (171), while in the intestinal compartment IgA levels remained unchanged. In mesenteric lymph nodes (MLNs), the intensive exercise training increased the proportion of T cells, mainly due to an increase in the Th cell subset, while decreased that of B cells, which may be associated with the lower levels of IgA found in salivary glands, given that IgA-producing memory B cells are normally activated in GALT structures and then migrate to the salivary glands (165). Nevertheless, running to exhaustion temporarily restored the Th cell proportion, probably due to their mobilization into the blood, as supported later by the findings reported in the **Article 5**. Besides these changes in cell proportions, the function of MLNs lymphocytes was also altered. The intensive training reduced the proliferative ability of T cells, in agreement with previously reported results (123,356), while increased the release of the proinflammatory cytokine interferon (IFN) γ and the production of IgG by MLN cells. The final ET increased the release of interleukin (IL) 2 and normalized the proliferative capacity of T cells. On the other hand, the intestinal barrier function was also impaired after intensive exercise. The intensive training downregulated the gene expression of claudin-4 and occludin in small intestine, in agreement with previous studies (198), while induced a more than three-fold increase in claudin-2 expression, a pore-forming claudin whose upregulation is normally associated with leaky-gut and the appearance of diarrhoea (201,202). In addition, running to exhaustion induced an immediate increase in zonula occludens-1 expression, matching with other studies (198–200). Despite all these changes in tight junction gene expression, when assessing the intestinal concentration of alpha-1-antitrypsin, a biomarker of gut paracellular permeability, only a trend to increase after running to exhaustion was found. Summarizing, **Article 2** demonstrates that intensive training for 5 weeks followed or not by a session of exhausting exercise, besides affecting systemic immunity, it also alters the mucosal immune system and the intestinal epithelial barrier function in Wistar rats.

Alterations on immune system have been described not only after prolonged periods of intensive exercise training, but also after a single session of intensive exercise (21,25). Then, we decided to use a rat model of acute exercise, i.e., a single bout of intensive exercise, both to assess changes in the mucosal immunity (fitting the first objective of the thesis) and to establish the role of dietary interventions with cocoa and cocoa fiber in this model (fitting the second objective of the thesis: evaluating the potential of nutritional strategies for the prevention of the detrimental effects of both acute and chronic intensive exercise on immune system). The results obtained with this model of intensive exercise are published in **Articles 3-4**. The model of acute exercise included, firstly, a week during which the animals were adapted to run on a treadmill and homogeneously distributed according to their ability to run into runner and sedentary groups and, inside these groups, into three subgroups according to the diet: reference, 10% cocoa and 5% cocoa fiber. The six groups had the same average in terms of ability to run. After distribution, animals underwent a 3-week no exercise washout period, and then, a second familiarization week was carried out. Finally, the exhausting exercise bout was performed and, in this case, we chose to collect the samples 16 h after exercise cessation to establish the changes that are not immediately restored and that could be responsible for the higher risk of infections observed in athletes, as done in previous studies (187,357,358).

The exercise-induced immune disruption is likely triggered by the oxidative stress induced by exercise, thus we hypothesized that a nutritional intervention containing polyphenols may help counteracting them. As widely explained in the introduction of this thesis, previous studies from our research group have demonstrated the antioxidant, immunomodulatory, and anti-inflammatory effects of a diet containing 10% cocoa (C10 diet) in preclinical models of immune-mediated diseases such as allergy (272,275,296), arthritis (247,270) and inflammation (285), as well as in healthy animals (290,306). Considering this background, we firstly hypothesised that the intake of the C10 diet could prevent the immune alterations induced by acute exercise (**Articles 3 and 4**). In addition, given that cocoa fibre per se has shown immunomodulating (301) and prebiotic (309) properties, we also aimed to assess the effects of a diet providing the same amount of fibre than the C10 diet on immune system in acutely exercised rats (**Articles 3 and 4**). The intake of cocoa fibre for 25 days exerted several positive effects in the intestinal compartment, such as preventing the exercise-induced increase in faecal humidity. Furthermore, although the CF diet did not modify the proportion of *Clostridium coccoides/Eubacterium rectale* or *Lactobacillus/Enterococcus,* it enhanced the caecal concentration of short chain fatty acids, preventing the reduction in propionic acid induced by acute exercise. Moreover, the CF diet up-regulated the small

intestine gene expression of toll like receptor (TLR) 7, which seems to be decreased in human peripheral blood mononuclear cells after marathon running (101). With regard to other immune compartments, the CF diet also prevented the acute exercise-induced decrease in salivary IgM. Surprisingly, cocoa fibre not only induced beneficial changes in the mucosal compartment, but also avoided the increase in ROS production by peritoneal macrophages induced by acute exercise, similarly as the C10 diet. These results, together with another study reporting reduced plasma levels of malondialdehyde after a similar intervention with cocoa fibre (256), suggest that the intake of a small amount of polyphenols, as such included in the fibre, may be enough for preventing oxidative stress. On the other hand, cocoa increased the small intestine weight and the number of bacteria in caecal content, but reduced the proportion of IgA-coated bacteria and *Clostridium coccoides/Eubacterium rectale*. Furthermore, the intake of the C10 diet normalized the changes induced by acute exercise in the proportion of Tαβ cell and Tγδ cell subsets in MLN and PPs, respectively. In addition, the C10 diet decreased the release of the proinflammatory cytokine IFN-γ by MLN lymphocytes, whose concentration was increased after the protocol of chronic intensive exercise, both alone and followed by a final session of exhausting exercise (**Article 2**), but not in the acute exercise model (**Article 4**). Overall, from **Articles 3 and 4**, we can conclude that both cocoa and cocoa fibre protect against the oxidative stress induced by a single session of exhausting exercise, although they modulate differently the intestinal immunity. Nevertheless, it remained to know the effect of C10 diet in a longer intensive training.

During the development of the second objective of the thesis, we also aimed to determine the effects of flavonoids in the exercise-induced immune alterations. For this, we conducted a study using a pure flavonoid: hesperidin. This flavanone is one of the most consumed polyphenols and has previously demonstrated immunoenhancing properties (338,340,342). Furthermore, recent preclinical (69) and clinical (331,332,359,360) studies have reported its ergogenic, antioxidant, anti-inflammatory and metabolism modulating effects. Considering this background, we hypothesized that hesperidin supplementation could prevent the immune alterations induced by intensive training and exhausting exercise in rats. In **Article 5**, we used the same intensive training approach than in **Article 2**. Throughout the 5 weeks of exercise training, hesperidin (200 mg/kg body weight) was administered by oral gavage three times per week based on a previous study showing the antioxidant and ergogenic effects of hesperidin on a similar exercise model (69). Hesperidin supplementation improved the exercise performance during the chronic intensive training, avoiding the decrease in running capacity observed after the resting days in the non-supplemented

Wistar rats, in agreement with previous preclinical (69,329) and clinical studies (259,361–364). In addition, hesperidin prevented the increase in circulating leukocytes induced by the final ET and avoided the higher secretion of IFN-γ by peritoneal macrophages observed in intensively trained rats, in agreement with previous studies (365,366), although it did not avoid the increase in TNF-α and IL-6 production in such rats. The results from **Article 5**, together with the protective effects of hesperidin on exercise-induced oxidative stress from previous studies (69), allow us to conclude that hesperidin may be a potential nutraceutical candidate for both enhancing exercise performance and preventing the exercise-induced immune alterations, which may result in normalizing the higher risk of infection observed in athletes during periods of intensive training and/or after exhausting exercise.

In the following experiments, based on the results of **Article 2**, the exercise programme was modified with the aim of intensify it. The exercise training, performed in the same treadmill but now with 5° of uphill, lasted for one more week (6 weeks in total). Moreover, the regular trainings were longer (25, 30, and 40 min instead of 20, 25 and 30 min) and more intense (70% of the maximum speed average achieved in the previous Monday ET instead of 60%). In addition, Lewis rats were used instead of Wistar rats for being an inbred strain more sensitive to immune changes. On the other hand, given that the number of study groups needed for evaluating a nutrition intervention was bigger than in the rat model setup carried out in **Article 2**, we just collected samples before (T group) and immediately after (TE group) performing the final ET.

The results of **Article 6** slightly varied from those observed in **Articles 2 and 5**, which could be due to the different rat strain used in these studies. For instance, although all trained rats had higher levels of plasma cortisol after running to exhaustion, the increase was larger in Wistar rats (**Article 5**) than in Lewis rats (**Article 6**). This difference in the endocrine response to intensive exercise may be due to the lower hypothalamic-pituitary-adrenal axis reactivity observed previously in Lewis rats (367,368). Lewis rats secrete lower levels of corticotropin releasing hormone than Wistar rats, which would make them less responsive to stress and might be responsible for the different effects on immune system in response to exercise observed in the present thesis. If further studies clarify these strain differences, Wistar rats may be more adequate than Lewis rats for exercise immunology studies, even though they are not inbreed and Lewis are more used in the immunology field. Anyway, **Article 6** allowed us to evaluate the influence of cocoa and to establish the potential synergistic effects between cocoa and hesperidin in intensively trained and exhausted rats, before and after performing a session of exhausting exercise. In this case, we decided to administer hesperidin

through the diet to mimic a dietary intake in humans (10% cocoa plus 0.5% hesperidin diet, CH diet), choosing the dosage from previous preclinical studies (342,369,370).

The dietary interventions with 10% cocoa (C10) or 10% cocoa and 0.5% hesperidin (CH) were performed in parallel to the exercise intervention, which means it also lasted for 6 weeks. In this case, surprisingly when considering the effects of **Article 5**, no improvement in exercise performance was observed in rats fed the CH diet. This could be due to different possible reasons. First, the dosage used may not be the optimal for achieving ergogenic effects. A diet containing 0.5% hesperidin has previously demonstrated beneficial effects on immune function (342), as well as in bone metabolism (369,370), however, it has never been used in an exercise immunology study. In **Article 6**, although the dietary intervention provided higher amount than that in the **Article 5** (3150 mg/kg per week versus 600 mg/kg per week), the intake rate was slower, since rats consumed the supplemented chow throughout the day, which probably result in a lower peak of hesperidin concentration in plasma, as previously observed in pharmacology studies (371). Furthermore, there could be some inhibitory interaction between hesperidin and the cocoa components. Last, the different effects of hesperidin on exercise performance observed in **Article 5** and **Article 6** may also be due to the differences in the exercise protocols and rat strains used. In fact, in the first approach (**Article 5**), hesperidin mainly avoided the performance decline after the resting days, while in the second approach (**Article 6**), such decline was not statistically significant.

The intake of the C10 diet in **Article 6** attenuated the increase in plasma cortisol concentration observed after the final ET, inducing a reduction in these hormone even in non-exercised rats, as previously described (372). In addition, the C10 diet promoted the abundance of Th cells in the spleen, contrary to what has been observed in the MLNs (**Article 4**), preventing the exhaustion-induced reduction in such cells, as previously reported in Lewis rats (286), but not in Wistar rats (271). Furthermore, the C10 diet enhanced the cytotoxic activity of NK cells and modified the concentration of serum Igs in both runner and sedentary rats. Most of the effects induced by the intake of the CH diet were also observed in the C10 groups, which suggests that cocoa is the responsible for these effects, again, probably because the method of administration and the dosage of hesperidin were not the optimal. Still, some changes were observed exclusively after the dietary intervention with the CH diet, such as the increased proportion of B cells in spleen and, consequently, the higher in vitro production of IgM by spleen lymphocytes. Nevertheless, the preventing effect on the exhaustion-induced alterations in spleen Th proportion seems to be due to cocoa.

Then, summarizing, from the effects observed in the C10 groups of **Article 6** it can be concluded that a dietary intervention with cocoa modulates systemic immune function, preventing some of the alterations induced by intensive exercise. Nevertheless, to better assess the efficiency of such intervention, a more challenged immunity as a result of a more intensive and longer exercise protocol may be needed. The effects observed in the CH groups of **Article 6** allow us to conclude that although a dietary intervention with cocoa and hesperidin can enhance immune function and prevent some of the alterations induced by exercise, such preventing effects are probably due to cocoa. We encourage further studies to clarify the optimal dose and administration method of hesperidin for preclinical studies aimed to study its impact on immunity, and specially, in the exercise context.

CONCLUSIONS

The results obtained from the current thesis led us to conclude that:

- Intensive training twice a day for two weeks followed by a session of exhausting exercise modifies the gut microbiota composition in both male and female Wistar rats, although most of the alterations depend on the rats' sex.

- Intensive training in female Wistar rats once a day for five weeks, alone or followed by a final session of exhausting exercise:

    - Disrupts the intestinal epithelial barrier integrity, by altering the gene expression of tight junction proteins in particular affecting claudins and occludin due to intensive training, and zonula occludens due to exhaustion.

    - Alters the function of mucosal-associated lymphoid tissue. Thus, intensive training provokes a reduction in salivary IgA whereas exhaustion enhances the caecal IgA, and both conditions affect the composition and cytokine secretion of mesenteric lymph nodes lymphocytes.

- A single session of intensive exercise in female Wistar rats is able to alter the oxidative stress, gut microbiota functionality and mucosal immunity. Particularly, it:

    - Increases the reactive oxygen species production by peritoneal macrophages and modifies the composition of caecal short chain fatty acids by reducing propionic acid production.

    - Influences immunoglobulin content in several compartments. In particular, it decreases the levels of IgG in plasma and IgM in salivary glands. Likewise, it modifies the lymphocyte composition in Peyer's patches and mesenteric lymph nodes.

- A dietary intervention with cocoa or cocoa fibre, in a model of a single session of intensive exercise, does not affect exercise performance but protects against oxidative stress, enhances the caecal short chain fatty acid production, prevents some changes in Peyer's patches and mesenteric lymph nodes lymphocyte composition, and decreases the production of proinflammatory cytokines. However, only cocoa fibre prevents the lower salivary IgM induced by exercise.

- An oral supplementation with hesperidin to rats submitted to an intensive training (once a day for five weeks followed by a final session of exhausting exercise) improves exercise performance. In addition, it enhances natural killer cell cytotoxicity and the proportion of phagocytic monocytes, attenuates the secretion of cytokines, prevents the leucocytosis induced by the final exhaustion, and increases the proportion of T cells, mainly Th cells, in several lymphoid compartments.

- Intensive training in female Lewis rats once a day for six weeks in an uphill treadmill induces an increase in plasma cortisol and some immune changes such as a higher granulocyte phagocytic activity and alterations in spleen cell phenotype and function.

- A dietary intervention with 10% cocoa in rats trained daily for six weeks in an uphill treadmill, does not improve exercise performance but prevents the plasma cortisol increase and some of the immune alterations induced by exercise. The inclusion of 0.5% of hesperidin to the 10% cocoa diet barely induce any additional effect. Both diets induce changes in immune system beyond those effects of training.

Overall, different models of intensive exercise have been applied and evidenced some immune impairments. Dietary interventions with cocoa, cocoa fibre and hesperidin have partially prevented these changes without affecting exercise performance.

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