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Immunomodulatory Effects Associated with Lactofermented Cherry Beverage Consumption in Rats

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Highlights

1. The development of a fermented cherry beverage using *Lactobacillus plantarum* is a promising solution for improving human health and diet.
2. The cherry-based fermented beverage boosted total antioxidant capacity in rats, increased melatonin and serotonin levels, and aided in the regulation of inflammatory processes.
3. The intake of lactofermented cherry beverage may confer health benefits by modulating the immune system.

Abstract: Cherry is a fruit which contains elevated amounts of antioxidant compounds, such as anthocyanins, pigments, and vitamins. Furthermore, it possesses high water, sugar, mineral, and indolamine contents. The general objective of this study was to characterise a cherry-based fermented beverage (the 'sweetheart' variety) and analyse the effects of its ingestion on (i) circulating serum levels of melatonin and serotonin, (ii) inflammatory response, and iii) serum total antioxidant capacity in rats (*Rattus norvegicus*). For cherry-based fermented beverage manufacturing, the cherries were washed, the stems and woody endocarps were removed, and ascorbic acid was added (to avoid enzymatic browning). After the homogenisation of the cherry fruit, lactic acid bacteria were inoculated, and the fermentation process was conducted for 36 h. The main bioactive compounds in the cherry beverage were characterised, as well as their total antioxidant capacity. Moreover, an in vivo assay was developed, in which rats ingested the fermented beverage ad libitum for seven days. The inflammatory mediators, the total antioxidant capacity, and the serum levels of melatonin and serotonin were measured. Based on these results, the intake of the cherry-based fermented beverage assayed in this study increased the total antioxidant status of rats, elevated the melatonin and serotonin levels in the serum, and improved the regulation of the inflammatory systemic processes.

Keywords: lactofermented cherry beverage; immunomodulatory effects; oxidative status; biogenic amines; functional food

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1. Introduction

The cherry is a reddish fruit, a drupe type, from the cherry tree of the *Rosaceae* family and the *Prunus* genus. It is characterised by a low caloric value and a high water content. It is rich in phytonutrients, phytochemicals, vitamins, sugars, and organic acids [1,2].

The anthocyanins and phenolic compounds found in cherries possess antioxidant properties and act as powerful free-radical scavengers [3,4]. Moreover, these molecules

have enormous potential to prevent or mitigate neural and behavioural age-related disorders [5,6]. In the last decade, numerous in vivo studies have reported that the consumption of cherries, particularly sweet cherries, not only increases antioxidant status [6,7] but also has beneficial effects on sleep quality and mood status. This is due to their high content of melatonin and its precursors, the neurotransmitter serotonin, and the amino acid tryptophan [8–10].

Melatonin is a low-molecular-weight indole (232.278 g/mol) that is ubiquitously present in all living organisms. Despite its simple structure, it is a pleiotropic molecule with several important biological properties [6,11]. This molecule has a high antioxidant power, neutralising '-OH'radicals. Regarding its intrinsic ability to purify free radicals, melatonin is capable of stimulating the activity and expression of other antioxidant systems, which establish an indirect form of action to reduce oxidative stress [6]. For its part, serotonin (176.2151 g/mol) is a monoamine found in small quantities in enteric nervous system neurones, platelets, and tryptaminergic neurones. It is also released by platelets in the inflammatory focus and may play a protective role against stress and oxidative damage derived from polymorphonuclear leukocyte function owing to its antioxidant capacity [12]. Tryptophan (204.23 g/mol) is an essential amino acid and a precursor of serotonin, melatonin, niacin, and quinurein, which are involved in important biological functions [13,14].

These three molecules, melatonin, serotonin, and tryptophan, play important roles in the regulation of biological functions, such as sleep, appetite, learning, memory, and mood [10,13,15]. Numerous investigations have revealed a close relationship between the mediators of immune responses (pro-inflammatory and anti-inflammatory cytokines) and these important molecules [16].

Jerte Valley cherries (Extremadura, Spain) present not only a high content of phytonutrients and phytochemicals [9,17,18] but also an important content of tryptophan [9], melatonin, and serotonin [17]; thus, obtaining cherry-enriched foodstuffs may be a successful strategy to make/produce natural foodstuffs with numerous health benefits for consumers.

Fermentation is a process that not only causes chemical changes but may also improve the nutritional and health properties of food components. Microbes play an important role in fermented foods by introducing new compounds that ultimately reach the gut [19]. Interestingly, many of these microbial species are closely related to probiotic strains, suggesting potential health benefits [20]. The use of lactic acid bacteria in the production of fermented beverages has been a long-standing practice in the dairy industry to enhance the functionality of traditional fermented products. However, recent studies have shown that lactic acid bacteria can also be used to produce plant-based fermented beverages, such as fruits, fruit juices, and vegetable analogues such as almond and soy milk. These plant-based alternatives have been developed to replicate the texture and flavour of traditional dairy-based fermented beverages [21,22].

Therefore, fermented foods serve as a significant dietary source of live microorganisms, providing a natural means of introducing beneficial bacteria into the organism. The presence of microbes in fermented foods may contribute to human health like probiotics do, thereby promoting overall well-being [19,23].

The primary aim of this study was to thoroughly examine a beverage derived from the lactic fermentation process of the 'Sweetheart' variety of sweet cherries, focusing on its functional and nutritional properties. Additionally, this study aimed to explore the potential health-promoting effects of regular consumption of a cherry-based fermented beverage and its implications for overall well-being. For this purpose, the effects of consuming the beverage ad libitum for 7 days on various physiological parameters were investigated. These parameters included the circulatory levels of melatonin and serotonin, total serum antioxidant activity, and inflammatory response in Wistar rats (*Rattus norvegicus*).

2. Materials and Methods

2.1. Vegetal and Animal Materials

Cherries (*Punus avium* L.) of the 'Sweatheart' variety, harvested at commercial maturity following the criteria used in the Valle del Jerte (Cáceres, Spain), were used in this study. These cherries were sourced from the central industry. This study was carried out on 10 male Wistar rats (*Rattus norvegicus*) aged 6 weeks, which were supplied by the Animal Service of the University of Extremadura (Spain). Rats were individually housed under controlled temperature conditions (20 ± 5 °C), with a diet based on 'Panlab' feed.

2.2. Selection of Strain Lactobacillus and Growth Curve

To narrow down the lactic acid bacteria in this study, we explored the strains available in our collection (the Department of Biotechnology and Sustainability (CICYTEX)) to identify the strains that were best suited to the production of lactofermented beverages.

Among the strains in our collection, four were selected for evaluation and testing. To reactivate the activity of the strains stored in deep freezing (−80 °C), *Lactobacillus paraplantarum*, *Lactobacillus plantarum*, and *Lactobacillus salivarius* were grown in MRS broth (Scharlab, Spain) for 24 h under total aerobiotic conditions, whereas *the Lactobacillus paracasei* strain was grown for 24 h in a microaerophilic atmosphere. After preculturing, all strains were diluted to concentrations of 5×10^4 and 10^5 CFU/mL in MRS broth, and their respective growth curves were studied in quintuplicate using a TECAN multiplate reader. Absorbance was measured at 600 nm every 40 min for 1440 min (24 h) with respect to the aeration conditions used in the precultures.

The maximum growth rate during the exponential phase of the growth curve was analysed to determine the growth rate of each strain. Data analysis involved calculating the growth rate of each strain during the exponential growth phase using the following formula:

$$\text{Maximum growth rate } (MGR) = \frac{(Y2 - Y1)}{(X2 - X1)} \quad (1)$$

2.3. Cherry Beverage

Lactobacillus plantarum was used as the bacterial strain for beverage preparation. This strain was grown in MRS broth at 30 °C for 24 h. After incubation, the bacteria were collected by centrifugation (10.000 g, 10 min at 4 °C). They were then washed in 50 mM potassium phosphate sterile buffer (pH 7.0) and resuspended in sterile distilled water to a final optical density of 620 nm (O.D. 620) of 2.5 (corresponding to a final number of cells approximately equivalent to 9.0 log colony forming units (cfu) mL^{-1}).

First, cherries were washed and pitted. Ascorbic acid was then added to prevent enzymatic browning prior to purée homogenisation and pasteurisation. Once the purée was cooled, the lactic acid bacterium strain (*L. plantarum)* was inoculated and fermented for 36 h at 25 °C, growing from 6.35 ± 0.46 log cfu g^{-1} until 9.83 ± 0.13log cfu g^{-1}.

2.4. Animal Treatment and Serum Collection

Rats (*Rattus norvegicus*) were tested under two different conditions: before the consumption of the cherry beverage (basal samples) and after the intake of the cherry beverage (treated samples). First, the rats consumed tap water ad libitum (basal) for 7 days. Tap water was then replaced with the cherry beverage, and the rats consumed this beverage ad libitum for 7 days (treated samples).

Blood samples (2 mL of blood) were drawn from rats before (basal serum) and after the intake of the cherry beverage (treated serum) at 8:00 a.m. and introduced into a tube containing serum separation gel. Before collecting blood, local anaesthesia was applied to the tail, and a cut was made 1 mm from the tip of the tail using a scalped blade. Blood flow was stopped by dabbing the tip of the tail. The blood samples were centrifuged at 300× *g* for 30 min at room temperature. The serum was then separated into aliquots and stored at −30 °C until analysis.

2.5. Functional Compounds Analysis

Determination and quantification of tryptophan in the beverage were carried out using an amino acid analyser Biochrom-30 (Biochrom Ltd., Cambridge Science Park, Cambridge, England), following the methodology described by [24]. The detection and quantification of serotonin, indole, and melatonin were determined using the chromatographic method described by [17]. The extraction of total anthocyanins was performed according to the method described by [25], and quantification was carried out according to the method described by [26]. Total phenols were determined by spectrophotometry, according to the method described by [27]. Antioxidant activity was evaluated according to the procedure described by [28].

For the determination and quantification of serum serotonin, a serotonin-ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany) was used according to the manufacturer's instructions. The total antioxidant capacity of the serum was evaluated according to the method described by [28] for hydrophilic samples.

2.6. Identification and Quantification of Inflammatory Mediators

Determination and quantification of interleukin (IL) 10, 6, and 8, as well as tumour necrosis factor alpha (TNF-α), were measured and quantified using a commercial ELISA kit (Rat Quantikine; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.7. Statistical Analysis

Results are expressed as means ± standard deviations. Statistical differences were calculated using the Student's t-test for paired values, which compares the different conditions. The significance level was set at $p < 0.05$. All analyses were performed using GraphPad Prism version 5.0, 2007 (GraphPad Software, San Diego, CA, USA) and OriginPro 9.0 (OriginLab Software, Northampton, MA, USA).

3. Results

3.1. Pre-Screening of Lactobacillus Strain Growth Curves and Maximum Growth Rate

As shown in Figure 1, all the lactic acid bacteria tested grew in less than 24 h, reaching high optical densities at 600 nm, and their behaviour up to and during exponential growth varied significantly. The results of this study showed that *L. paracasei, Lactobacillus salivarius*, and *L. paraplantarum* had a longer latency period before exponential growth (over 400 min). In contrast, *L. paraplantarum* and *L. salivarius* showed similar behaviours in terms of maximum growth rate during the exponential growth phase, with the latter strain showing a maximum growth rate that was approximately 7% higher, as shown in Table 1. *L. plantarum* had higher optical densities and MGR. Interestingly, a significant difference in growth patterns was observed between *L. paraplantarum* and *L. plantarum*. These strains presented dissimilar exponential growth curves, with *L. paraplantarum* having a latency time that was 150 min longer than that of *L. plantarum*, and the MGR was approximately 30% lower.

Table 1. The maximum growth rate of lactic acid bacteria during the exponential phase was calculated according to Equation (1).

Strain	MGR (DO 600 nm/min)
Lactobacillus plantarum	0.0059575
Lactobacillus paraplantarum	0.00417
Lactobacillus paracasei	0.002775
Lactobacillus salivarius	0.004415

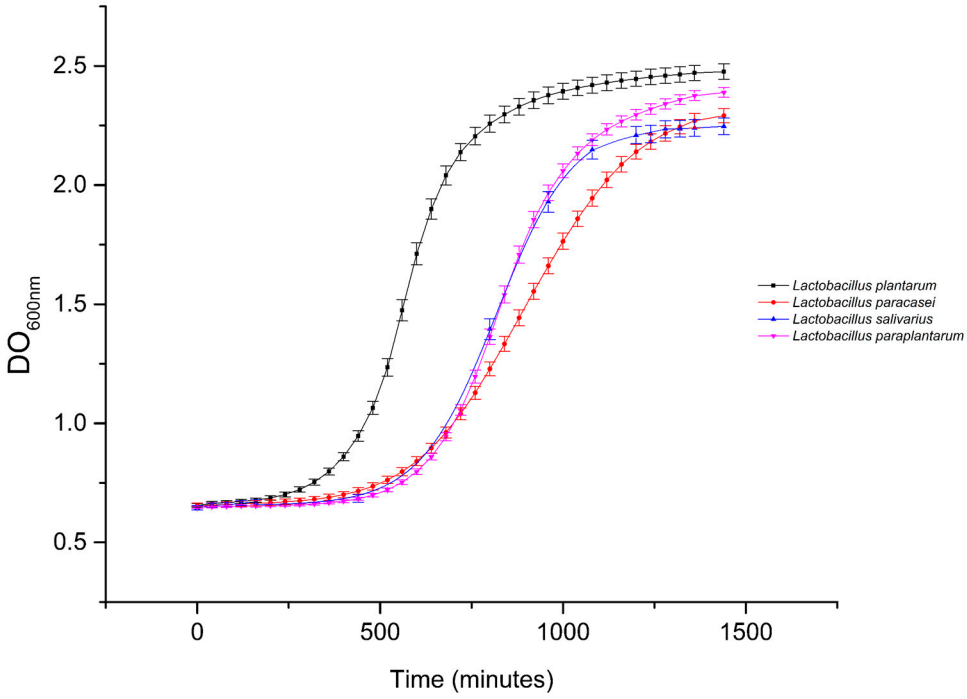

Figure 1. Comparison of growth curves of acid lactic acid bacteria.

3.2. Characterisation of the Functional Cherry Beverage

Table 2 shows the bioactive compounds found in the cherry beverage after functional characterisation. It should be noted that the detected concentration of melatonin was 7.80 ± 0.91 ng per 100 g, and the total antioxidant capacity of the cherry beverage found after analysis was 37.65 ± 2.4 mmol Trolox L^{-1}.

Table 2. Characterisation of the cherry beverage obtained by lactic acid fermentation. Values represent means ± standard deviations.

Bioactive Compounds	Concentration
Tryptophan (mg/100 g beverage)	10.15 ± 0.33
Serotonin (ng/100 g beverage)	14.97 ± 1.07
Anthocyanin (mg/100 g beverage)	7.97 ± 0.10
Melatonin (ng/100 g beverage)	7.80 ± 0.91
Total phenolic compounds (mg/100 g beverage)	331 ± 14
Total antioxidant capacity (mmol Trolox L^{-1})	37.65 ± 2.4

Compounds identified in blood serum and their total antioxidant capacity.

The serum levels under the basal conditions and after the 7 day administration of the functional cherry beverage are shown in Figure 1. The circulatory melatonin (Figure 2A) and serotonin (Figure 2B) levels increased significantly ($p < 0.05$) after the cherry beverage intake.

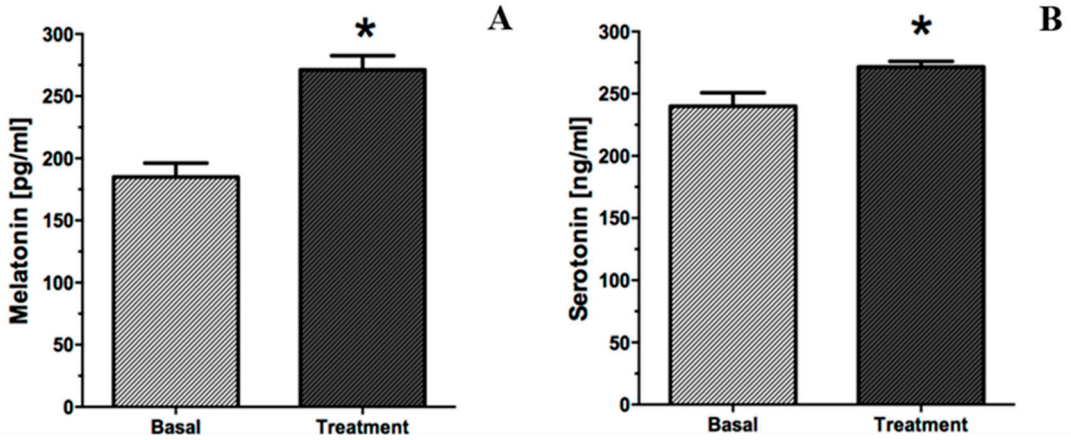

Figure 2. Melatonin (**A**) and serotonin (**B**) serum levels were measured before the intake of the cherry beverage (basal) and after a 7 day period of consumption of the cherry beverage (treatment) obtained from sweet cherries (*Punus avium* L.) in 10 male young rats. Each value represents the means ± standard deviations of ten determinations, performed in duplicate. * Statistically significant with respect to basal conditions ($p < 0.05$).

Figure 3 shows the inflammatory response mediators detected and quantified in serum: interleukins and TNF-α (Figure 3A), as well as the total antioxidant capacity (Figure 3B). The levels of pro-inflammatory mediators, both TNF-α and IL-8, decreased ($p < 0.05$) after the intake of the cherry beverage, whereas the concentration of anti-inflammatory mediators increased, particularly the levels of IL-10, which were significantly ($p < 0.05$) higher than those obtained under basal conditions.

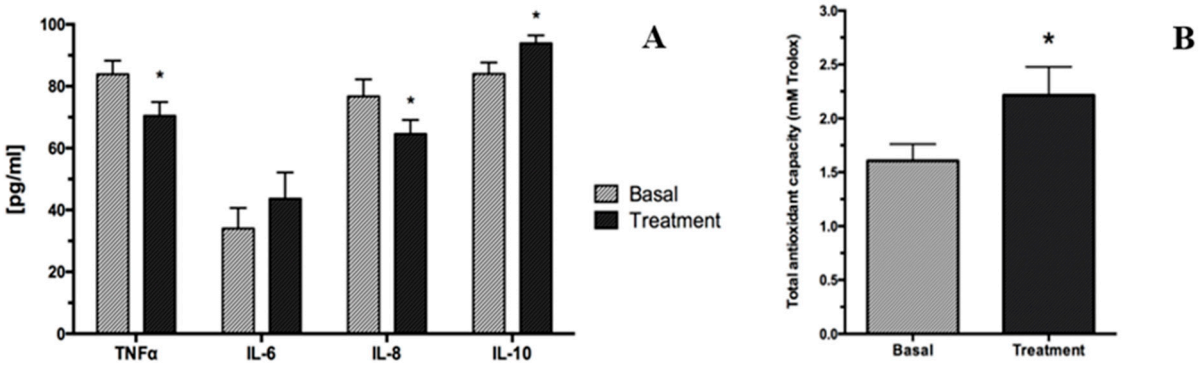

Figure 3. Interleukin and TNF-α quantification in blood serum (**A**) and total antioxidant capacity (**B**) before the intake of the cherry beverage (basal) and 7 days after the administration of the beverage obtained from sweet cherries (*Punus avium* L.) (treatment) in 10 young male rats. Each value represents the mean ± standard deviation of ten determinations, performed in duplicate. TNF-α: tumour necrosis factor alpha; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10. * Statistically significant with respect to basal conditions ($p < 0.05$).

A remarkable increase ($p < 0.05$) was observed in the total antioxidant capacity of the serum (Figure 3B) due to the ingestion of the cherry functional product.

4. Discussion

4.1. Immunomodulatory Properties of the Lactofermented Cherry Beverage

Extensive research has consistently demonstrated the antioxidant properties inherent to phytochemical compounds found abundantly in fruits and vegetables [29], and it is assumed that the consumption of these foods exerts a protective effect on consumer health [6]. In fact, several international organisations have recommended increasing the fruit and

vegetable intake to five or more servings per day to achieve an adequate proportion of antioxidants, thereby reducing the incidence of certain diseases [30].

In recent years, the amino acid tryptophan, the neurotransmitter serotonin, and indole melatonin have been identified as important bioactive compounds in plants. This may have implications for human health because of their potent antioxidant activity against disorders and diseases related to free-radical production [31,32]. The levels of tryptophan, serotonin, and melatonin quantified in the studied cherry beverage are in accordance with the range obtained by other authors for fresh cherries and processed cherries [9,18,33]. Tryptophan has gained special interest in the diet because it is involved in the regulation of numerous physiological processes, which exert direct and indirect actions through its conversion into serotonin and melatonin indole [33]. The presence of melatonin is important, because it may play a role in regulating aging [31].

The concentration of anthocyanins found in the present cherry beverage was comparable to that obtained in other processed plants, in which beneficial effects on health related to the anthocyanin content have been demonstrated [34].

Ref. [35] have demonstrated the impact of phenolic compounds on human health, mainly due to their implication in a wide range of biological activities (antimicrobial, antioxidant, or antimutagenic effects, among others). For this reason, the high content of phenolic compounds detected in the studied cherry beverage could be of great physiological interest.

The values obtained for the total antioxidant capacity did not exactly match the results obtained by other authors. In fact, great variability between different authors has been found when this parameter is evaluated. Analytical factors, such as methodology, oxidation substrate, and extraction method, and intrinsic variables, such as agronomic, varietal, or technological factors, influence the results. Therefore, it is not possible to establish a direct comparison with previous studies.

The circulatory melatonin levels increased in rats after the ingestion of the cherry beverage. Similar results were reported by [36,37], who demonstrated that the intake of a cherry nutraceutical product (Spanish patent no. ES 2,342,141 B1) for 10 days enhanced the indole levels in both ringdoves (*Streptopelia risoria*) and rats (*Rattus norvegicus*). This increase may be caused not only by a direct consequence of melatonin but also by the increased levels of tryptophan and serotonin quantified in the cherry beverage studied. In fact, serotonin and tryptophan, direct precursors of melatonin, increased notably after intake of the cherry beverage. These results may have important health-related implications because of the wide spectrum of biological functions in which these molecules are involved [38,39].

Previous studies have shown that fluctuations in serum melatonin concentrations are strongly linked to the antioxidant capacity of the blood in birds and mammals, including humans [40]. Based on the antioxidant properties of melatonin and its precursors, in the current study, the high total antioxidant capacity observed after the ingestion of the cherry beverage could be closely related to the increase in these three molecules after the intake of the beverage. However, other bioactive compounds present in cherries, such as phenols, flavonoids, isoflavonoids, phytosterols, and phytic acid, are commonly associated with beneficial health effects [41,42]. In the current study, the high levels of phenolic compounds and anthocyanins reached after the intake of the cherry beverage could have contributed, at least in part, to the increased total antioxidant capacity observed in rats after the 7 day period of beverage consumption.

Only a slight increase in the concentration of IL-6 was detected among the mediators of the immune response. This may be due to the fact that interleukin is not only considered a mediator of the inflammatory response but also can act as an anti-inflammatory mediator [43]. Another factor that may explain the lack of significant differences in IL-6 levels is the circadian rhythmicity of interleukins. In addition, it has been shown that the concentration of IL-6 reached after the intake of a cherry-enriched product depends on different factors, such as sampling time. [36,37] also demonstrated that the consumption of a cherry-based product decreased the levels of IL-8 and TNF-α in rats and ringdoves, resulting in different outcomes, depending on the time of measurement.

The anti-inflammatory effects of the cherry fruit are of vital importance and have been investigated in animal models of arthritis [44]. Animals fed high doses of anthocyanins from cherries showed a significant decrease in TNF-α and prostaglandin E2 levels. The importance of cherries in the reduction of inflammatory responses has been demonstrated.

IL-10 is an anti-inflammatory cytokine involved in important processes, such as the proliferation of mast cells and the prevention of IFN-γ production by NK cells [31,45]. The results obtained in the present study revealed that the intake of a functional beverage obtained from sweet cherries positively modulated the levels of IL-10 in rats.

In general, the intake of a cherry beverage rich in melatonin, serotonin, and the precursor of both, the amino acid tryptophan, promoted an increase in the serum levels of both indolamines. This increase was probably linked to an increase in antioxidant capacity and the ability to modulate the different inflammatory mediators. These effects my accur through direct mediation of melatonin or through the modulation of other physiological mediators, without ruling out the role of the polyphenols contained in the beverage.

4.2. Feasibility of Lactobacillus Plantarum for Industrial Scale-Up

According to our findings (Table 1 and Figure 1), among the strains studied, *L. plantarum* may be a more suitable candidate for the industrial production of fermented fruit drinks because of its short adaptation time to growing conditions, maximum growth rate in the exponential phase, and achievement of high optical densities in both commercial MRS and cherry-based formulated culture media. These findings are in line with a large number of studies that have used *L. plantarum* as a microorganism to design fermented fruit drinks. For example, to optimise the strain, [22] took advantage of genetic engineering techniques to modify the *L. plantarum* strain, optimising its metabolism and improving its properties as a starter culture. This optimization aimed to improve the total antioxidant activity by increasing polyphenols and glutamine in the final fermented fruit-based beverage. [21] highlighted the sensory improvement and shelf life provided by *Lactobacillus plantarum* in the design of a fermented apple beverage.

According to [46], for industrial scale-up, the most important parameters to be met by the chosen strain are tolerance to specific environmental conditions, such as pH and temperature, as well as the ability to perform the desired trophic activities. In this sense, as explained throughout this research, the cherry-based medium did not hinder *L. plantarum* from reaching high densities (10^9 UFC/mL), and the starting pH was not an impediment to the development of the strain.

In addition, the temperature used (30 °C) is a mild temperature, which does not entail a very high input energy cost and its respective associated carbon footprint in the industrial production of the beverage. This beneficial aspect is highlighted as important by [47] and [48] in their studies on the economic and ambient effect of inputs used in the food and beverage manufacturing sector.

To sum up, this research has demonstrated that the intake of the fermented cherry beverage developed here causes an elevation of the total antioxidant status of rats and an augmentation of the melatonin and serotonin levels in the serum, facilitating the regulation of systemic inflammatory processes. This indicates that the cherry beverage produced via lactic acid fermentation can serve as a functional beverage with immunomodulatory properties. The existence of natural antioxidants in fermented cherries could be the reason for their capacity to enhance the body's antioxidant status, while an increase in melatonin and serotonin levels may positively impact sleep regulation and mood. Furthermore, the beverage's ability to modulate the systemic inflammatory response suggests its potential health benefits in the form of a reduced risk of chronic inflammatory diseases.

5. Conclusions

The intake of the cherry-based fermented beverage developed in this study increased the circulatory levels of melatonin and serotonin, as well as the antioxidant status of

Rattus norvegicus. In addition, this beverage participated in the modulation of systemic inflammatory processes in the animal model studied.

This study provides novel insights into the functional properties of *Lactobacillus plantarum* and its potential for industrial scale-up in the production of cherry beverage lactofermentates to enhance health and well-being if incorporated into the human diet. Nevertheless, this study was limited to a specific range of Lactobacillus species, and further research is needed to explore the probiotic properties of other species in the genus or other genera with similar metabolic capabilities. In addition, an in-depth study of metabolic pathways and regulatory processes is needed to improve on-demand *L. plantarum* homeostasis to improve both functional and sensory properties and optimal fermentation parameters.

Author Contributions: J.D.-A. performed the experiments. J.R.-P., S.M. and M.G. conducted the investigation. M.G. and J.D.-A. were responsible for the software. B.N.-P. and S.M. carried out the validation. J.D.-A. was responsible for the resources. M.G. and J.D.-A. were responsible for data curation. B.N.-P., J.R.-P., S.M. and M.G. performed the writing—original draft preparation, while review and editing were conducted by M.G., B.N.-P., S.M. and J.D.-A. Visualization was handled by B.N.-P. and J.R.-P. Supervision was provided by M.G. and J.D.-A. Project administration was carried out by M.G. and Jonathan Delgado Adamez. Funding acquisition was managed by J.D.-A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Animal Experimentation Committee of the University of Extremadura (registration number: 128/2023 and approved 28 September 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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

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