



## Low-molecular-weight transfer factor molecules improve the expression of CD<sup>2+</sup> leukocytes in male finishing pigs

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### Abstract

The small intestine is a major site of immune defense in pigs. Low-molecular-weight transfer factor (TF) molecules are known to play an essential role in regulating immune responses. This study aimed to investigate the effects of oral administration of TF on the expression of CD<sup>2+</sup> leukocytes in the small intestine of male finishing pigs. Eighteen 18 male finishing pigs were randomly assigned to control, 300 mg/day TF, and 600 mg/day TF. TF was administered orally for 30 days. The expression of CD<sup>2+</sup> leukocytes was determined by IHC. Results showed that oral administration of TF significantly increased the expression of CD<sup>2+</sup> leukocytes in the small intestine of pigs. The increase was observed in all three segments of the small intestine but was most pronounced in the ileum for 600 mg/day, with highly significant differences ( $P < 0.01$ , Duncan) and the response curves duodenum =  $1.2114 / (1 + 3.7576e^{-6.4119x})$  CD<sup>2+</sup> cells/mm<sup>2</sup>, jejunum =  $1.34258 / (1 + 2.36566e^{-2.94002x})$  CD<sup>2+</sup> cells/mm<sup>2</sup>, ileum =  $1.751185 / (1 + 4.16359e^{-2.543x})$  CD<sup>2+</sup> cells/mm<sup>2</sup>. Oral administration of transfer factor (TF) significantly increased the levels of CD<sup>2+</sup> leukocytes in the duodenum, jejunum, and ileum of male finishing pigs. The increase was most pronounced in the ileum, with the 600 mg/day dose producing the greatest effect. These results suggest that oral administration of TF could be an effective strategy to improve small intestinal immune function in male pigs, especially at higher doses.

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### Introduction

Due to the increasing rise in bacterial resistance and the risk of inducing drug resistance that this preventive antibiotic management represents, the swine industry is looking for new probiotic, prebiotic, or symbiotic additives that improve health and protect pigs from diseases (1,2). This practice poses public health problems, and the swine industry is also looking for additives that follow the principles of One Health (3). At the same time, in the area of pig production, the quality of meat presupposes sustainable management that, via healthy feeding, allows for the generation of an excellent

quality final food product for humans. In this regard, transfer factors, nano molecules that act as immunomodulators (4), represent a nutritional alternative that can be explored to increase the animals' immune response. The intestinal epithelium is a fundamental part of the immune system (5), and by improving it (6), some productive parameters, including weight gain and feed conversion, can also be improved (7). According to Gutiérrez (8), although there is still a lack of reproducible scientific evidence on the use of transfer factors and their mechanism of action, there is no doubt that they have a biological effect that should be used in all clinical and productive areas. Obtaining information on

immunomodulatory foods, such as transfer factors and their effect on the T lymphocyte group, can provide new pathways in the search for nutraceutical foods that can be used in animal feed to reduce antibiotics in animal nutrition. Industrial developments in human nutrition as a nutraceutical (4,9-11), as well as the response shown in animals (8,12,13) and currently Yu *et al.* (14) suggest that they have a satisfactory response when used as a dietary supplement in farm and companion animals (15). Similarly, CD<sup>2+</sup> cells have potential biomedical applications since  $\gamma\delta$  T cells have been used in human cell therapy to treat diseases such as cancer (16) and viral infections and have been characterized in some respects for pigs (17). Therefore, studies on  $\gamma\delta$  T cells in pigs can provide crucial information on the ability of these cells to fight diseases and how they can be used to develop new cell therapies in humans. However, it is necessary to know if they have contraindications as a supplement that could affect their expression, as antibiotics do (18). The objective was to identify changes in the number of CD<sup>2+</sup> T cells in the small intestinal epithelium of finishing pigs after consumption as a supplement of the immunomodulator transfer factor. Transfer factors comprise low-weight molecules derived from leukocytes that could transfer specific immunity to naïve T lymphocytes (19). Although their chemical nature and mechanism of action are not fully understood, their efficiency has been demonstrated in humans in various diseases (20). The immunomodulator transfer factor (21) is also known as (DLE) dialyzable leukocyte extract because it was the first source from which they were obtained. Their effect has also been demonstrated in patients with sepsis, showing that DLE dialyzable leukocyte extract called transfer factors suppress the production of TNF- $\alpha$  induced by LPS (lipopolysaccharides/endotoxin) in peripheral blood and monocytes. Another mechanism of action is the increase of cell-mediated immunity and gamma interferon, especially in natural killer (NK) cells. For example, effects have been found in treated mice that exhibited a moderate inflammatory response that decreased over time, and in the end, a lung architecture with minor alteration was observed. An effect was also found in turtles, where it caused an increase in T lymphocytes in the spleen (8), in dogs affected by parvovirus (12), and in dairy cows affected by gastrointestinal infections (13). Based on the industrial developments of the use of transfer factors isolated from bovine colostrum (11) already for nutritional use and oral administration, with applications in dairy cattle for the prevention and treatment of gastrointestinal diseases (13), and the subsequent developments of extraction from chicken egg (4) or the combinations of several transfer factors (9) for oral administration in humans (22) as a dietary supplement without known contraindications with verifiable results on immunomodulation (23), with the use of nano factors due to their small molecular size, it is possible to think of uses in animal production as preventive nutrition in all species. Although both conventional T cells ( $\alpha\beta$  T) and  $\gamma\delta$  T cells

express CD<sup>2</sup>, it has been shown that  $\gamma\delta$  T cells have a higher density in the intestine (24). They also participate in the NK cell expression phenotype in pigs (CD<sup>2+</sup>CD<sup>4</sup>CD<sup>5</sup>CD<sup>8 $\alpha$ +</sup> cells) (25). In some cases, there are unique perforin expression profiles related to CD<sup>2+</sup> in pigs, which can be a helpful tool when treating an infection (26,27).

This research aimed to explore the potential of transfer factors as a nutraceutical supplement in animal feed to improve the animals' immune response. In addition, it seeks to obtain information that helps to provide information on potential applications of transfer factors and their action on CD<sup>2</sup> T cells present in the intestine, including cell therapy for diseases such as cancer and viral infections.

## Materials and methods

### Ethical approval

All experimental procedures followed the guidelines suggested by the International Guiding Principles for Biomedical Research Involving Animals (28) and were approved on May 18, 2018, by the Ethics Committee on Animal Experimentation of the Universidad Cooperativa de Colombia.

### Transfer factor

The preparation methodology of the transfer factor from egg yolk 4LIFE<sup>®</sup> is extensively delineated in the patent by Hennen *et al.* (4). This methodology involves identifying a non-mammalian female source animal, such as a hen. A characteristic antigen of a specific pathogen is introduced into the source animal, which can be accomplished via injection, ingestion, or other methods. The source animal is then allowed to lay eggs. These eggs are collected, and the transfer factor is extracted from them. This extraction can be performed using ultrafiltration, chromatography, or electrophoresis techniques. The transfer factor is then purified to eliminate other egg constituents, such as larger molecular weight proteins, with ultrafiltration being utilized in this instance. The transfer factor is now prepared for incorporation into a composition or device for administration to a mammalian or non-mammalian subject. Alternatively, the transfer factor can be directly generated in the egg of a non-mammalian source animal. This can be achieved by exposing the egg to the antigenic agent (e.g., via injection of the antigenic agent into the egg). The transfer factor can then be extracted from the egg using the abovementioned methods. The preparation method from colostrum is described in the Wilson *et al.* patent (11). The antigen-specific excreted transfer factor can be obtained by collecting colostrum or milk from a lactating mammal with immunity to the antigen of interest by ultrafiltration. The colostrum or milk is then processed to remove materials that interfere with transfer factor efficacy. The resulting product is a transfer factor, which can be used directly or concentrated and purified further.

## Animals

On a farm, eighteen commercial crossbred male pigs (Landrace Belga x Large White) with an average weight of 55 kg and 14-week age were used in a completely randomized design with three treatments: control (0 mg/day), T1: (300 mg/day), and T2: (600 mg/day) of 4LIFE® transfer factor per animal. Transfer factor was obtained from the extract of bovine colostrum (11) and egg yolk (4) by ultrafiltration. The experiment lasted 30 days, with an alpha error of 0.01. The TF was administered daily during the entire experiment. The diet did not exceed the recommendations for finishing pigs (FEDNA). The diet was not changed during the experiment, so no acclimatization was required. The animals were under clinical observation for ten days before the start of the experiment, and their health was monitored throughout the study.

## Sample collection

The small intestinal epithelial tissue was collected for immunohistochemistry analysis of CD<sup>2+</sup> expression (29). The tissue was collected as described by Ciro (30) and Itza-Ortiz (31), superior to methods involving pressure, washing, and subsequent fixation. The tissue was collected by tying the ends with nylon sutures and injecting 10% formalin directly into the lumen without removing the intestinal contents. The tissue was then placed in sterile containers with 10% formalin for fixation and preservation.

## Immunohistochemistry

Immunohistochemistry staining was performed using specific antibodies to the target protein CD<sup>2+</sup>. A peroxidase-based detection system was used to reveal the antigen-antibody reaction. Three tissue sections were evaluated for each sample: duodenum, jejunum, and ileum, taking on the characteristic color brown (32). Each section was photographed at 400 magnifications using a Leica DM500 microscope (33). The number of intraepithelial lymphocytes per millimeter square was calculated using Leica LAS EX 3.4 DVD272 software and the freeware IMAJEJ software (34).

## Statistical analysis

The data were analyzed using SPSS software (version 25) (35). The results were presented as mean ± SD. One-way ANOVA was used to analyze the significance of differences between groups, with Duncan as the post hoc test. Different lowercase letters were used to denote significant differences between groups. A  $P < 0.01$  was considered statistically significant.

## Results

Clinical evaluation revealed that pigs had mild diarrhea at the beginning of the trial, which decreased towards the end without medication. It is important to note that this diarrhea

is common in this farm in these growth periods, and it did not represent a significant epidemiological value of disease. After staining (Figure 1), the labeled cells were identified and counted (Figure 2). The density of CD<sup>2+</sup> cells showed statistically significant differences ( $P < 0.01$ , Duncan's test) between the 600 mg and 300 mg doses and the control group, but not between the 300 mg/day and 600 mg/day doses in the duodenum and jejunum (Figure 3). The ileal values were consistently higher than the other two segments, and the 600 mg/day values showed significant differences compared to the 300 mg/day and control (Figure 3).

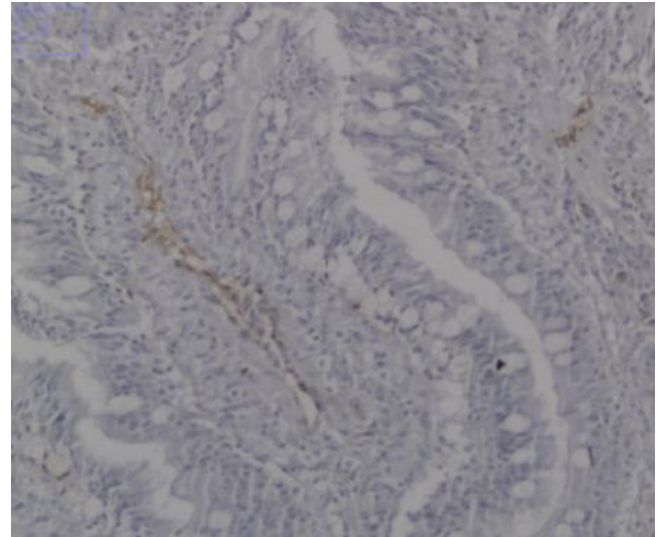


Figure 1: Presence of CD<sup>2+</sup> cells in the small intestine of pigs; note the characteristic brown color of the immunohistochemistry staining.



Figure 2: Identification and counting of cells using IMAJE software for the same tissue.

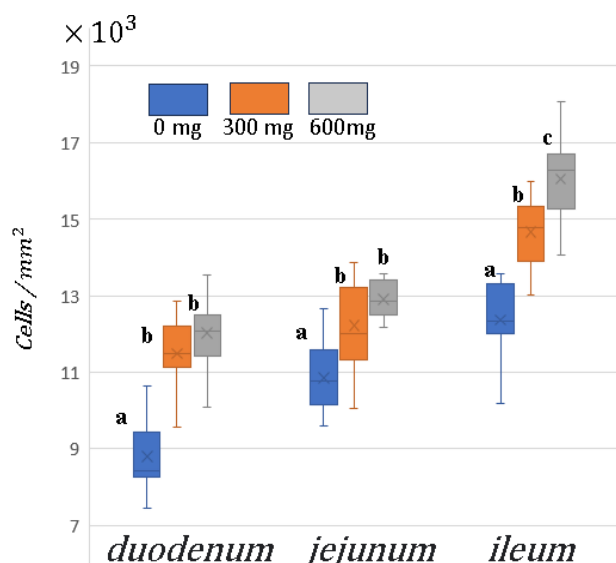


Figure 3: Response of CD2<sup>+</sup> leukocytes to different doses in pigs' three segments of the small intestine. Different letters in the same segment represent highly significant differences (P<0.01, Duncan).

Logistic regression analysis showed that the logistic curve was a good estimator of the CD2<sup>+</sup> response to different TF doses in each small intestine segment. This suggests that the CD2<sup>+</sup> response to TF is a non-linear function of the dose. The next are the calculated curves function for the response (Table 1).

Table 1: Logistic regression for expression CD2<sup>+</sup> (cells / mm<sup>2</sup>)

Logistic regression	Coeff of Determination (r <sup>2</sup> )	Correlation Coeff (r)	RMSE
CD2 duodenum=1.2114/(1+3.7576e <sup>-6.4119x</sup> )	0.730133	0.854478	879.24
CD2 jejunum=1.34258/(1+2.36566e <sup>-2.94002x</sup> )	0.522636	0.722936	836.92
CD2 ileum=1.751185/(1+4.16359e <sup>-2.543x</sup> )	0.731921	0.855524	948.48

## Discussion

The results of this study showed that oral administration of transfer factors (TF) as a food additive for 30 days significantly increased the density of CD2<sup>+</sup> cells in the small intestine of pigs, a response similar to that reported by Rekiel for other additives (36) and are consistent with those reported by Vega-Lopez and Telemo (37). This finding is consistent with other studies that have reported beneficial effects of TF on immune function, including increased levels of CD2<sup>+</sup> cells in peripheral blood and in the small intestine of canine (12) calves (13) laying hens (15,38) and humans (39-41). This could strengthen a circulating cell type, as Hirt *et al.* (42) and Stepanova (29) described. Therefore, the higher levels in the small intestine after oral delivery of transfer factors may be related to circulating levels, as some cancer treatments using interstitial dialyzable leukocyte extracts (43) increase CD2<sup>+</sup>

Figure 4 presented the CD2<sup>+</sup> expression in the three segments of the small intestine of pigs after oral administration of three doses of transfer factors 99% confidence.

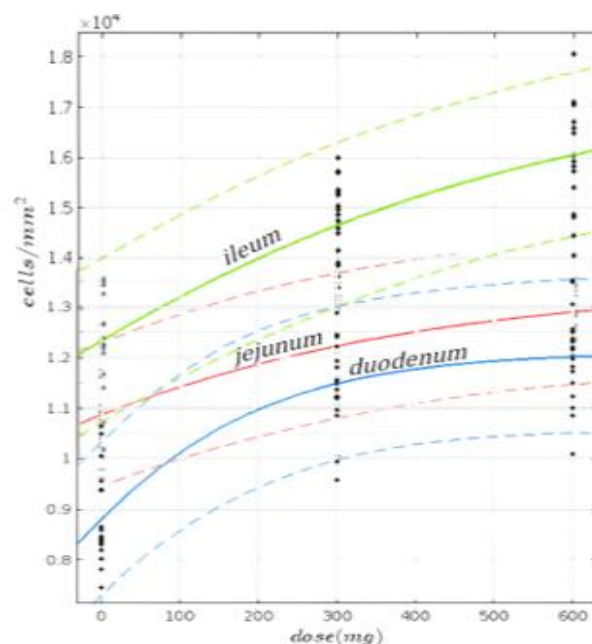


Figure 4: CD2<sup>+</sup> expression in the three segments of the small intestine of pigs after oral administration of three doses of transfer factors 99% confidence.

levels in peripheral blood in addition to CD4<sup>+</sup> CD8<sup>+</sup> levels (44). It is known that mature pigs normally have an unusual phenotype of CD2<sup>+</sup> CD4<sup>+</sup>CD8<sup>-</sup> cells in peripheral blood, as Gerner *et al.* (25) reported in a study found that this cell type can also be found in the intestine. Rothkötter (45) reported that these cells can be expressed in the intestine when they are in the blood.

On the other hand, the porcine large intestine contains lymphoid clusters (46), and the high levels of response in the ileum suggest that the ileum may be an essential site for the action of TF in the small intestine. TF are complex proteins that contain a variety of immune factors, including lymphokines, cytokines, and chemotactic factors (47-49), and these factors can act to stimulate innate and adaptive immune responses, which may contribute to the increase in CD2<sup>+</sup> cells in the small intestine. CD2<sup>+</sup> cells are a type of lymphocyte that plays a vital role in the immune response.



These cells can help regulate the immune response by identifying and eliminating pathogens and promoting wound healing.

## Conclusion

Oral administration of 300 mg of transfer factor significantly increased the levels of CD<sup>2+</sup> leukocytes in the duodenum, jejunum, and ileum finishing pigs, with even more significant increases observed for the 600 mg dose in the ileum only.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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## جزينات عامل النقل منخفضة الوزن الجزيئي تحسن التعبير لـ CD<sup>2+</sup> كريات الدم البيضاء في ذكور الخنازير

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### الخلاصة

الأمعاء الدقيقة هي موقع رئيسي للدفاع المناعي في الخنازير. ومن المعروف أن جزينات عامل النقل منخفضة الوزن الجزيئي تلعب دوراً أساسياً في تنظيم الاستجابات المناعية. تهدف هذه الدراسة إلى تقييم تأثير إعطاء عن طريق الفم على التعبير لـ CD<sup>2+</sup> كريات الدم البيضاء في ذكور الخنازير. تم تعيين ثمانية عشر ١٨ من خنازير الانتهاء من الذكور بشكل عشوائي للسيطرة، ٣٠٠ ملغ/يوم، و ٦٠٠ ملغ/يوم. تم إعطاء جزينات عامل النقل منخفضة الوزن الجزيئي عن طريق الفم لمدة ٣٠ يوماً. تم تحديد التعبير لـ CD<sup>2+</sup> كريات الدم البيضاء من قبل IHC. وأظهرت النتائج أن تناول جزينات عامل النقل منخفضة الوزن الجزيئي عن طريق الفم أدت إلى زيادة كبيرة في التعبير لـ CD<sup>2+</sup> كريات الدم البيضاء في الأمعاء الدقيقة للخنازير. لوحظت الزيادة في جميع الأجزاء الثلاثة من الأمعاء الدقيقة ولكنها كانت أكثر وضوحاً في الدقاق لـ ٦٠٠ مجم/يوم، مع اختلافات كبيرة للغاية ( $> 0.01$ ) ومنحنيات الاستجابة الاثنى عشر =  $1/2114$  (٣,٧٥٧٦+١) / (٦,٤١١٩ س) سي دي ٢ + خلايا/مم ٢، الصائم =  $1/34258$  (٢,٣٦٥٦٦+١) / (٢,٩٤٠٠٢ س) سي دي ٢ + خلايا/مم ٢، اللفانفي =  $1/1751185$  (٤,١٦٣٥٩+١) / (٢,٥٤٣ س) سي دي ٢ + خلايا/مم ٢. تناول جزينات عامل النقل منخفضة الوزن الجزيئي عن طريق الفم أدت إلى زيادة كبيرة في مستويات التعبير لـ CD<sup>2+</sup> كريات الدم البيضاء في الاثنى عشر، الصائم، والدقاق في ذكور الخنازير. كانت الزيادة أكثر وضوحاً في الدقاق، حيث أنتجت جرعة ٦٠٠ مجم/يوم أكبر تأثير. تشير هذه النتائج إلى أن تناول جزينات عامل النقل منخفضة الوزن الجزيئي عن طريق الفم يمكن أن يكون استراتيجية فعالة لتحسين وظيفة المناعة المعوية الصغيرة في ذكور الخنازير، وخاصة عند الجرعات العالية.