Evaluation of Lactoferrin Transport *In Vitro* Using the Everted Intestinal Sac Method and Its *In Vivo* Effects on Growth and Liver Color (L*) in Rats

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Abstract. This study aimed to evaluate the absorption and transport of lactoferrin *in vitro* using the everted intestinal sac method and to determine its *in vivo* effects on growth performance and liver color (L*) in rats. The research material included bovine lactoferrin, $FeSO_4 \cdot 7H_2O$, 0.9% NaCl solution and male albino rats (*Rattus norvegicus*) aged 4-5 months with body weights ranging from 200–350 g (*in vitro*) and male or female rats 7 day aged with body weights 10-15 g (*in vivo*) among other materials. The *in vitro* experiment demonstrated that lactoferrin absorption increased and reached a plateau at 60–70 mg/80 mL of 0.9% NaCl solution (14.53-14.92%), declined at higher concentrations, and was highest in the duodenal segment (15.38%), followed by the jejunum (11.59%) and ileum (6.89%). Time-course analysis revealed a progressive increase in absorption up to 75 minutes (17.66%) followed by a slight decline, indicating an active and saturable absorption mechanism. In the *in vivo*, rats receiving a combination of $FeSO_4 \cdot 7H_2O$ and lactoferrin showed the greatest body weight gain (1.61 g) and darker liver coloration (L* = 34.41), reflecting enhanced iron utilization and storage. These findings indicate that lactoferrin enhances intestinal iron absorption and improves physiological responses when combined with $FeSO_4 \cdot 7H_2O$. The integration of *in vitro* and *in vivo* results highlights lactoferrin's potential as a functional bioactive protein for improving iron bioavailability and promoting growth.

1 Introduction

Iron is one of the essential trace elements required for various physiological functions, including oxygen transport, energy metabolism, and enzymatic reactions. Iron deficiency remains one of the most prevalent nutritional disorders worldwide, particularly affecting infants, children, and women of reproductive age. Despite the widespread use of conventional iron supplements such as ferrous sulfate ($FeSO_4 \cdot 7H_2O$), their bioavailability is often limited, and gastrointestinal side effects may reduce compliance [1,2]. Therefore, improving iron absorption and minimizing adverse effects are important goals in nutritional intervention strategies.

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Lactoferrin (Lf), an iron-binding glycoprotein naturally present in milk and various mucosal secretions, has gained increasing attention as a functional protein with multiple biological activities. Lactoferrin can reversibly bind two ferric ions per molecule, enabling it to play a dual role in both iron sequestration and delivery [3]. Recent studies have reported that lactoferrin enhances intestinal iron absorption by modulating the expression of iron transporters and maintaining gut epithelial integrity [4,5]. In addition, lactoferrin has been shown to promote growth performance, modulate immune responses, and protect against oxidative stress [6].

In vitro absorption models, such as the everted intestinal sac method, have been widely used to investigate nutrient and drug transport across the intestinal epithelium under controlled conditions. This model allows for the direct evaluation of intestinal permeability and active absorption mechanisms in different intestinal segments, including the duodenum, jejunum, and ileum [7]. Complementary *in vivo* studies using animal models, such as rats, provide a more comprehensive understanding of nutrient utilization, growth response, and physiological impact, particularly with respect to liver metabolism and systemic iron status [8].

In this study, lactoferrin transport and absorption were evaluated *in vitro* using the everted intestinal sac method to determine its uptake across intestinal segments as well as its absorption kinetics under various concentrations and time intervals. In addition, an *in vivo* study was conducted to assess the effects of lactoferrin, $FeSO_4 \cdot 7H_2O$, and their combination on growth performance and liver condition in rats. Collectively, these experiments aim to elucidate the role of lactoferrin in enhancing iron absorption and its associated physiological benefits in iron supplementation. However, not much research has been done on in vitro with inverted intestinal sac as well as in vivo.

2 Material and Methods

2.1 Material

Bovine lactoferrin (≥95% purity) was obtained from a certified biochemical supplier. Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), sodium chloride (NaCL) (0.9% NaCl solution), and other analytical-grade reagents were purchased from Merck (Germany). Infant formula milk with the same composition but different sources of iron.

Male Wistar rats (*Rattus norvegicus*) aged 4-5 months and with body weights ranging from 200–350 g were used for *in vitro* research, meanwhile, for *in vivo* research, male and female rats 7 day aged with body weights 10-15 g were selected randomly. The feed for rats used in this study refers to the standards set by the American Institute of Nutrition 1993 (AIN-93). All experimental procedures were conducted in accordance with ethical guidelines approved by the Institutional Animal Care and Use Committee.

2.2 *In Vitro* Study: Everted Intestinal Sac Method

The *in vitro* transport of lactoferrin was evaluated using the everted intestinal sac method adapted from [9] and modified by researcher. The first step in the in vitro method using the inverted intestinal sac is to fast the rats for 20-24 hours and provide them with only water ad libitum. The subsequent study used sections of intestine inverted so that the villi were on the outside. Both ends of the small intestine were tied and filled with a 0.9% NaCl solution, known as serous fluid (one ml for every 5 cm of intestine). The small intestine was then placed in a tube containing lactoferrin in a 0.9% NaCl solution, known as mucous fluid. During the experiment, the entire small intestine was submerged in the mucous fluid and kept

saturated with oxygen (100 bubbles per minute). The temperature was maintained at 37°C, and the mice were agitated continuously. Absorption was read at 525 nm using an Atomic Absorption Spectrophotometer (AAS). To calculate the percentage (%) absorption, divide the amount absorbed by the amount ingested and multiply by 100%.

To determine the highest percentage of lactoferrin that can be absorbed by the small intestine, 24 male Wistar rats, were used, then divided into 3 groups of 8 rats each. Each group was then tested with lactoferrin levels of 10, 20, 30, 40, 50, 60, 70, and 80 mg, respectively, dissolved in 80 ml of buffer solution (0.9% NaCl). Determination of the small intestine segment that can absorb the largest lactoferrin, 3 male Wistar rats were used, 70 mg of lactoferrin was dissolved in 80 ml of buffer solution (0.9% NaCl), then the small intestine segment (duodenum, jejunum, ileum) that could absorb the largest amount was determined. Determining the time required for the small intestine to absorb the largest amount of lactoferrin, 3 male Wistar rats, were used. 70 mg of lactoferrin was dissolved in 80 ml of buffer solution (0.9% NaCl). The time required (15, 30, 45, 60, 75, and 90 minutes) for the maximum amount of lactoferrin to be absorbed was determined.

The *in vitro* transport of lactoferrin was evaluated using the everted intestinal sac method adapted from [9]. After the rats were euthanized, the small intestine was excised and rinsed with cold physiological saline. Segments of duodenum, jejunum, and ileum were isolated, everted, and filled with 80 mL of 0.9% NaCl solution containing lactoferrin at varying concentrations (10, 20, 30, 40, 50, 60, 70, and 80 mg). Each sac was incubated at 37 °C in an oxygenated medium, and samples were collected at different time intervals (15–90 min). The concentration of lactoferrin absorbed into the mucosal fluid was determined spectrophotometrically and expressed as mg per 80 mL of 0.9% NaCl solution.

2.3 *In Vivo* Study

Determination of the minimum number of samples based on the Federer formula, namely 6 rats per group. Fiftly rat pups and their mother (10 rats) were used. They were divided into 5 groups of rat pups and 2 mother rats, and were given five different infant formula respectively i.e. (1) $FeSO_4 \cdot 7H_2O$, (2) lactoferrin, (3) combination of $FeSO_4 \cdot 7H_2O$ + lactoferrin, (4) control (standard diet only), and (5) placebo. $FeSO_4 \cdot 7H_2O$ and lactoferrin were used as the source of iron (Fe). During the experiment the rat pups also got regular milk from their mothers which were fed by AIN 93 diet. After 28 days of intervention, the rats were executed and liver was taken color (L*) analysis. The rats were weighed once a week. The rats weight gain was determined by calculating the weight difference.

2.4 Statistical Analysis

All experiments were conducted in triplicate. Data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) to determine significant differences among treatments at a 95% confidence level ($p < 0.05$). Results were expressed as the mean ± standard deviation (SD).

3 Result and Discussion

3.1 *In Vitro* Lactoferrin Absorption under Different Concentrations

The *in vitro* absorption of lactoferrin using the everted intestinal sac model showed a concentration-dependent increase in lactoferrin uptake into the mucosal solution (Table 1). Lactoferrin absorption (%) increased and reached a plateau at 60–70 mg/80 mL, then

decreased at 80 mg/80 mL. These results indicate that absorption efficiency increases with concentration up to an optimal level, beyond which saturation may occur.

This trend is consistent with previous findings indicating that protein-mediated transport of lactoferrin is saturable and concentration-dependent, suggesting the involvement of receptor-mediated endocytosis in the intestinal epithelium [5,10]. Lactoferrin receptors, which are primarily located on enterocytes, play a key role in regulating intestinal iron absorption through specific binding and internalization [4]. The observed plateau and decline at higher concentrations may reflect receptor saturation or competitive inhibition among lactoferrin molecules.

Table 1. Lactoferrin absorption at various concentrations (mg/80 mL NaCl 0.9%).

Concentration lactoferrin in mucosal solution (mg/80 ml NaCl 0.9%)	Absorption (%)
10	8.54±0.91^{e}
20	9.51±0.63^{d}
30	11.47±0.49^{c}
40	11.88±1.06^{bc}
50	12.45±0.17^{b}
60	14.53±0.28^{a}
70	14.92±0.97^{a}
80	11.59±0.47^{c}

Note:
* Average of three replicates
** The same letters in the same column indicate results that are not significantly different

3.2 Lactoferrin Absorption in Different Intestinal Segments

Significant differences were also observed in lactoferrin absorption among intestinal segments. The duodenum exhibited the highest absorption value (15.38%), followed by the jejunum (11.59%) and the ileum (6.89%) (Table 2). This gradient suggests that the duodenum is the primary site of lactoferrin uptake, which is consistent with its physiological role in mineral absorption, including iron.

Previous studies have demonstrated that lactoferrin receptors (LfR) are most abundantly expressed in the duodenal region, where iron absorption is tightly regulated [11,12]. Moreover, the duodenum's acidic microenvironment and active transport mechanisms facilitate the release and uptake of iron bound to lactoferrin, thereby enhancing overall bioavailability. The lower absorption observed in the ileum may be attributed to decreased receptor density and reduced transport activity [7].

Table 2. Lactoferrin absorption across intestinal segments in rats

Intestinal Segment	Absorption (%)
Duodenum	15.38±0.65^{a}
Jejunum	11.59±0.79^{b}
Ileum	6.89±0.44^{c}

Note:
* Average of three replicates
** The same letters in the same column indicate results that are not significantly different

3.3 Absorption Kinetics over Time

Time-course analysis revealed that lactoferrin absorption increased progressively up to 75 minutes, reaching a maximum uptake of 17.66%, followed by a slight decline at 90 minutes (15.40%) (Table 3). The absorption curve displayed a typical kinetic pattern, — consisting of an initial phase of rapid uptake, a plateau phase, and a subsequent decline, possibly due to metabolic turnover or saturation.

Similar kinetic profiles have been reported by [13], who observed that lactoferrin uptake in Caco-2 cell models peaked between 60 and 90 minutes, suggesting an energy-dependent transport process. The subsequent decrease may also result from protein degradation or diffusion equilibrium within the mucosal solution. These findings further support the conclusion that lactoferrin absorption is an active, time-sensitive process that stabilizes once transport capacity reaches equilibrium.

Table 3. Lactoferrin absorption at different incubation times

Time (min)	Absorption (%)
15	8.16 ± 0.37^{f}
30	11.28 ± 0.45^{e}
45	14.40 ± 0.79^{d}
60	16.90 ± 0.30^{b}
75	17.66 ± 1.08^{a}
90	15.40 ± 0.66^{c}

Note:
* Average of three replicates
** The same letters in the same column indicate results that are not significantly different

3.4 *In Vivo* Growth Performance in Rats

In vivo evaluation of growth performance showed that rats supplemented with a combination of $FeSO_4 \cdot 7H_2O$ and lactoferrin exhibited the highest weight gain (1.61 g), which was significantly higher than that of the control (1.26 g) and placebo (1.11 g) groups (Table 4). Both $FeSO_4 \cdot 7H_2O$ and lactoferrin administered individually also improved growth compared to placebo, although their effects were less pronounced than those observed with the combination treatment.

These results suggest a synergistic effect between lactoferrin and ferrous sulfate in enhancing iron utilization and promoting growth. This synergism may arise from lactoferrin's ability to stabilize iron in a bioavailable form while protecting against oxidative stress induced by free iron ions [6,14]. In addition, lactoferrin supports gut health and nutrient absorption by maintaining epithelial integrity and modulating intestinal microbiota [3,5].

Table 4. Average body weight gain of rats

Treatment	Weight Gain (g)
$FeSO_4 \cdot 7H_2O$	1.27 ± 0.09^{b}
Lactoferrin	1.30 ± 0.08^{b}
$FeSO_4 \cdot 7H_2O$ + Lactoferrin (mix)	1.61 ± 0.27^{a}
Control	1.26 ± 0.13^{b}
Placebo	1.11 ± 0.11^{c}

Note:
* Average of ten replicates
** The same letters in the same column indicate results that are not significantly different

3.5 Liver Color (L*) as an Indicator of Iron Status

Liver color measurements revealed that the placebo group exhibited the highest L* value (37.98), indicating a lighter coloration, whereas $FeSO_4 \cdot 7H_2O$ and lactoferrin-treated groups showed lower L* values (34.40–34.77), corresponding to darker coloration (Table 5). The control group (35.71) intermediate L* value. The darker liver color observed in the treatment groups is associated with indication higher iron deposition and metabolic activity within hepatic tissues.

Previous studies have used liver color as an indirect indicator of iron storage and heme synthesis [8,11]. Lactoferrin's facilitation of iron transport to the liver enhances ferritin formation, resulting in the accumulation of iron-containing pigments and a corresponding decrease in L* values [15]. These results confirm that combined supplementation of $FeSO_4 \cdot 7H_2O$ and lactoferrin effectively improves systemic iron status and utilization.

Table 5. Liver Color Parameter (L*) Of Rats Under Different Treatments

Treatment	L* Value
$FeSO_4 \cdot 7H_2O$	34.40 ± 1.57^{c}
Lactoferrin	34.77 ± 2.46^{b}
$FeSO_4 \cdot 7H_2O$ + Lactoferrin (mix)	34.41 ± 2.57^{bc}
Control	35.71 ± 2.60^{a}
Placebo	37.98 ± 2.99^{b}

Note:
* Average of ten replicates
** The same letters in the same column indicate results that are not significantly different

3.6 Integrated Interpretation of *In Vitro* and *In Vivo* Findings

The integration of *in vitro* and *in vivo* findings provides a comprehensive understanding of lactoferrin's functional role in iron metabolism. The *in vitro* data clearly demonstrated concentration-dependent, segment-specific, and time-limited absorption of lactoferrin across the intestinal wall, indicating an active and saturable transport mechanism. Correspondingly, the *in vivo* experiments confirmed that lactoferrin, particularly when combined with $FeSO_4 \cdot 7H_2O$, enhances growth performance and supports hepatic iron storage.

These complementary results suggest that lactoferrin acts both as an iron-binding carrier and as a modulator of iron absorption, thereby ensuring efficient utilization while minimizing oxidative side effects [5,14]. This dual mechanism highlights the potential application of lactoferrin as a functional bioactive component in nutritional formulations aimed at preventing iron deficiency and promoting healthy growth.

4 Conclusion

This study demonstrated that lactoferrin plays a significant role in enhancing intestinal iron absorption and improving physiological responses *in vitro* and *in vivo*. The *in vitro* experiments revealed that lactoferrin absorption increased and reached a plateau at 60–70 mg/80 mL, whereas was highest in the duodenum segment, and peaked within 75 minutes of incubation, indicating an active and saturable transport mechanism.

In the *in vivo* study, supplementation with lactoferrin, particularly when combined with $FeSO_4 \cdot 7H_2O$, significantly improved weight gain and enhanced liver color (L*), indicating greater iron deposition and metabolic activity. Collectively, these findings suggest that

lactoferrin not only facilitates iron transport across the intestinal epithelium but also enhances systemic iron utilization, thereby promoting growth and maintaining liver function.

The integration of *in vitro* and *in vivo* results supports the potential application of lactoferrin as a biofunctional protein for improving iron bioavailability and as a complementary agent in iron supplementation strategies. Further studies are warranted to elucidate the underlying molecular mechanisms, including transporter regulation and antioxidant defense pathways, to strengthen the understanding of lactoferrin's nutritional and therapeutic benefits.

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