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CALCIUM AND VITAMIN D_2 ABSORPTION AND EFFECT ON NITROGEN UTILIZATION OF MILK

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ABSTRACT

Micronutrient fortified milk was developed and its bioavailability using *in-vivo* (rats) and *in*-vitro gastrointestinal simulated method was determined. In *in-vivo* studies, rats fed with fortified milk powders and its effect on nitrogen utilization indices were determined. Results showed that both calcium citrate and calcium phosphate showed similar absorption in all treatment groups *viz.* calcium, vitamin D_2 and calcium+vitamin D_2 groups. Vitamin D increased calcium absorption when fortified singly and in combination with both calcium salts. The weight of rats increased after every interval of week in comparison to day 0. The calcium and vitamin D fortified group showed significantly ($p<0.05$) highest protein efficiency ratio followed by vitamin D fortified group, calcium fortified group and control. Similar trend observed in net protein utilization and digestibility coefficient, however, both nutrient fortified group showed significantly ($p<0.05$) higher biological value than other three groups. Vitamin D has positive effect on protein digestibility indices; calcium has no or slight effect, whereas, when fortified in combination rats showed higher nitrogen utilization.

Keywords: *in-vitro* digestibility; *in-vivo* studies; nitrogen utilization indices; calcium; vitamin D_2

INTRODUCTION

Many studies carried out to check the effect of vitamin D supplementation on human metabolism. The presence of vitamin D receptors in most human tissues indicate the extraskeletal role of vitamin D **(Paul *et al.*, 2024)**. The literature confirms its role in calcium, potassium, iron and zinc and other minerals absorption, utilization and balance **(Kaushik *et al.*, 2014)**. It helps in optimizing skeletal muscle function **(Boland 2011)**; improve muscle strength **(Bischoff-Ferrari *et al.*, 2006)**; prevent falls **(Kiely *et al.*, 2017)**; activates protein kinase **(Olmos-Ortiz *et al.*, 2015)**, reduce pulvewave velocity, and improve arterial stiffness **(Chen *et al.*, 2020)**. It also enhance leydig cells performance and increase production of testosterone in men **(Holt *et al.*, 2020)**, attenuates lung injury via stimulating epithelial repair, reducing epithelial cell apoptosis **(Zheng *et al.*, 2020)**, metabolic syndrome and atherosclerosis **(Colak *et al.*, 2020)**.

Skeletal muscles have receptors for vitamin D and it maximizing muscle activity **(Boland, 2011)**. It impairs proximal muscle function and is through to pre-dispose falls in elderly. An improvement in muscle strength and movement was recorded when vitamin D level rose from 4 to 16ng/ml and continued to improve upto 40ng/ml **(Bischoff-Ferrari *et al.*, 2006)**.

Vitamin D is bound to vitamin D binding protein in blood and carried it to the liver, kidneys, intestine and other target tissues. Vitamin D low level and vitamin D binding protein expression level have negative outcomes during pregnancy, such as preterm delivery and restricted fetal growth **(Kilpatrick *et al.*, 2020)**. A person is considered as deficient in vitamin D when its blood 25(OH)D concentration is below 75nmol/L and severely deficient when below 25nmol/L **(Sempos *et al.*, 2018)**.

Bioavailability of proteins represents the amount of peptides and amino acids absorbed through small intestine. Despite the rapid and dynamic evolvement of research into food proteins, there is still a knowledge gap regarding their bioavailability for systemic circulation to targeted organs. Beside the digestive actions of endogenous proteases and peptidases, chemical and nutritional compositions of the food matrix and their physical forms (e.g., liquid, puree, gel, solid) are also critical factors that can influence the interactions, digestibility, bioavailability of calcium and vitamin D and proteins in fortified milk **(Sun *et al.*, 2020)**. Vitamin D receptor and membrane associated rapid response steroid binding protein found in the cell membrane bind 1,25$(OH)_2$D and initiate the activation of numerous pathways involving various protein kinase **(Olmos-Ortiz *et al.*, 2015)**.

Charoenngam, (2019) reported that 100 crore people worldwide have vitamin D deficiency. **Cashman (2019)** reported that more than 20% population has circulating 25(OH)D concentration <25nmol/L. Infants, young children and women of child bearing age were shown to be at highest risk. Vitamin D 800IU/day supplementation with calcium can prevent risk of falls. **Kiely (2017)** recommended 5 to 15 µg/day to achieve 25(OH)D targets of >25-50nmol/l (10 to 20ng/ml).

Nutrient deficiencies are generally occurs in combinations like vitamin D and calcium deficiencies are observed in same individuals **(Kaushik and Arora, 2017)**. All nutritionist reported that dairy products are superior in calcium retention; indeed, 65% of all ingested calcium absorbed from these sources **(Kaushik *et al.*, 2014a)**. However, the consumption of milk is declining in industrialized countries leading to inadequate calcium intake **(Rana *et al.*, 2003)**. This has opened new avenues in the development of calcium-fortified milk to deliver nutritionally dense milk. Calcium in milk is more easily absorbed by the intestine that the calcium from the vegetables and cereals. Phytates (present in cereals, beans and pulses), oxalates (present in leafy vegetables), long chain fatty acids and dietary fibers can reduce the bioavailability of calcium by forming insoluble calcium complexes **(Singh *et al.*, 2007)**.

Vitamin D consumption may not only fulfill the recommended dietary allowance of vitamin D but also increase the bioavailability of calcium and phosphorous **(Ross *et al.*, 2011)**. Health agencies reported association of calcium and vitamin D and have synergistic effect on skeleton health **(Laird *et al.*, 2010)**.

Bioavailability is a critical feature in the assessment of the role of micronutrients in human health and milk is an effective delivery vehicle for fat-soluble vitamins. However, data on the bioavailability of micronutrients (endogenous or synthetic) from dairy products in humans are very limited **(Sachdeva *et al.*, 2015a)**. *In vitro* models based on human physiology developed as simple, inexpensive and reproducible tools to predict the bioavailability of different food components **(Sachdeva *et al.*, 2015b)**.

In this study, developed the vitamin D and calcium fortified milk **(Kaushik *et al.*, 2015a; Kaushik *et al.*, 2015b)** absorption of added nutrients using cellulose membrane permeability *in-vitro* method was carried out. *In-vivo* studies were also carried out to determine the effect of fortification on nitrogen utilization of milk.

MATERIALS AND METHODS

Chemicals and Reagents

Calcium salts *viz*. Calcium phosphate dibasic and calcium citrate terahydrate; nitric acid and lanthanum chloride procured from Himedia, Mumbai, India. Vitamin D_2, α-amylase (EC 3.2.1.1), human pancreatic lipase (EC 3.1.1.3), colipase, cholesterol esterase (EC 3.1.1.13), phospholipase A2 (EC 3.1.1.4), mucin, bovine serum albumin, pepsin (2080 units per mg of protein), pancreatin and taurocholate salts were purchased from Sigma Chemical Co (Madrid, Spain). Ethanol, hexane, acetonitrile, methanol, (HPLC grade) were obtained from Carlo Erba (Madrid, Spain).

In vitro study

The micronutrient fortification was carriedout of toned milk *viz.* calcium at 600 ppm by two calcium salts (Calcium citrate and calcium phosphate, respectively) and vitamin D 15 µg/L singly and in combination with both salts of calcium and *in-vitro* bioavailability was determined by simulated gastrointestinal conditions with slight modifications. Dialysis tube membrane made up of modified cellulose with 16 mm diameter and 12-14 thousand dalton cut-off limit was cleaned with milli Q water and placed it in boiling milli Q water for 5 min. Then transfed it to clean dialysis tube in a 20 % alcohol aqueous solution (at 4 °C) for 10 min. Finally, the dialysis tube was cleaned with milli Q water 3 times. The one side of cellulose membrane was sealed and from second side 15 ml milk was poured and simulated digestion secretions added at similar concentration as in digestive system at similar conditions and maintain 37 ºC **(Sachdeva *et al.*, 2015b)**.
Vitamin D_2 and calcium content of retentate and permeate were determined by HPLC and AAS and digestibility of the added nutrients was determined using simulated gastro-intestinal conditions.

Calculation

The bioavailability of the nutrients calculated from the amount of the nutrient (vitamin D_2 or calcium) that had passed the dialysis membrane proportional to the total nutrient (vitamin D_2 or calcium) content of the sample (eq. 1).

$$\text{Bioavailability (\%)} = \frac{D}{C} \times 100 \quad \text{…….. eq. 1}$$

where,
D = vitamin D_2 or calcium content in the dialysate and
C = vitamin D_2 or calcium content of sample

Analysis of added nutrients

Calcium content was analyzed as per method described in **Kaushik *et al.*, (2023)**. Accurately measured 5ml of milk sample and transfer in a silica crucible. Put silica crucibles on hot plate for charring and then placed it at 600 ºC in muffle furnace for 16 h. After ashing, add lanthanum chloride 100 µl/100 ml (5 %) and then add 1 ml of nitric acid (69% concentrated) and diluted to 1000 times with milli Q water. For vitamin D analysis, milk samples were saponified and then extracted using n-hexane. Then, hexane was evaporated and volume made up to 1 ml by HPLC mobile phase and analyzed at wavelength 254 nm using HPLC **(Kaushik *et al.*, 2014b)**.

In-vivo study

The effect of fortification on nitrogen utilization was carried out on rats of Wister Strain. Rats were procured from Animal breeding Facility, National Institute of Pharmaceutical Education and Research, Mohali, India with an initial mean weight 38.89 g with mean age of 28 days. The study was approved by Ethical Committee of ICAR-National Dairy Research Institute and conducted in its Small animal house facilities. Forty rats were divided into equal five groups and acclimatized for 7 days. Five rats groups were fed on five different diets during experimental period of 4 weeks. Group 1 was nurtured on protein free synthetic diet (100 %). Group 2 was nurtured on milk lyophilate (1/3) and synthetic diet (2/3). Group 3 was nurtured on vitamin D_2 fortified milk lyophilate (1/3) and synthetic diet (2/3). Group 4 was nurtured on calcium phosphate fortified milk lyophilate (1/3) and synthetic diet (2/3). Group 5 was nurtured on both nutrient fortified milk lyophilate (1/3) and synthetic diet (2/3).

Nitrogen bioavailability of fortified milk in rat models

The calcium phosphate salt selected for *in-vivo* studies because it showed higher absorption than calcium citrate. The effect of added nutrients on nitrogen absorption and utilization from fortified milk was determined. The fixed amount of diet supplied to rats every day and after each day amount of diet consume noted down. Urine and droppings of rats were collected daily, percentage of nitrogen absorbed (eq. 2), and retained (eq. 3) was calculated on weekly basis for 28 days.

$$\text{Nitrogen absorbed} = \frac{(N_I - N_D)}{N_I} \times 100 \quad \text{…… eq. 2}$$

$$\text{Nitrogen retained} = \frac{N_I - (N_D - N_U)}{N_I} \times 100 \quad \text{…… eq. 3}$$

Where,
N_I = Total Nitrogen Intake (g)
N_D = Nitrogen in droppings
N_U = Nitrogen in urine

Nitrogen utilization indices

Nitrogen absorption and retention are related to protein quality indices. For conversion of nitrogen into protein 6.35 factor was used.

Protein efficiency ratio (PER)

The PER was determined by ratio of increase in rats weight and protein consumed (eq. 4)

$$\text{PER} = \frac{\text{Increase in rat weight (g)}}{\text{Nitrogen content} \times 6.35 \text{ (g)}} \quad \text{…… } eq.4$$

Digestibility coefficient (DC)

The DC is the percentage of nitrogen absorbed from ingested food source only. To calculate it, a control with nitrogen free diet group is required. The DC was calculated by eq. 5

$$\text{Digestibility coefficient} = \frac{[N_I - (N_{DP} - N_{DPF})]}{N_I} \times 100 \quad \text{……. e.q 5}$$

Where,
N_I = Total Nitrogen Intake (g)
N_{DP} = Nitrogen in droppings of protein diet group
N_{DPF} = Nitrogen in droppings of protein free diet group

Biological value (BV)

Biological value represents the retention of nitrogen from ingested food source only. The body was released nitrogen in droppings and urine of protein free diet are subtracted from nitrogen in droppings and urine of protein feed group, respectively. It was calculated as per e.q. 6

$$\text{Biological Value} = \frac{(N_{DP} - N_{DPF}) - (N_{UP} - N_{UPF})}{N_I - (N_{DP} - N_{DPF})} \times 100 \quad \text{……. e.q 6}$$

Where,
N_I = Total Nitrogen Intake (g)
N_{DP} = Nitrogen in droppings of protein diet group
N_{DPF} = Nitrogen in droppings of protein free diet group
N_{UP} = Nitrogen in urine of protein diet group
N_{UPF} = Nitrogen in urine of protein free diet group

Net protein utilization (NPU)

Net protein utilization of dietary protein is the product of digestibility coefficient and biological value divided by 100.

$$\text{Net protein utilization} = \frac{\text{Digestibility coefficient x Biological value}}{100}$$

Measurements

Nitrogen content of feed, feces and urine was analysed using Kjeldhal method (Kjeldhal digestion assembly, Vapodest 40 C. Gerhardt, UK Ltd., Brackley, Northants) as described by **AOAC (2005)**. The percentage nitrogen in the sample was calculated as follows:

$$\% N = \frac{(T-B) \times N \times 1.401}{\text{Sample weight (g)}}$$

Where,
T= ml acid required for sample titration
B= ml acid required for blank titration
N= normality of acid used for titration
The 6.38 conversion factor for nitrogen to protein used during calculations.

Data analysis

The results were compiled and tabulated in Microsoft excel 2015 (Microsoft Corp., Redmond, United States). Statistical analysis and descriptive analysis carried out

through its data analysis pack. Data analyzed as per single way ANOVA and using it CD value calculated at 95 % confidence level **(Chawla *et al.*, 2021)**.

RESULTS AND DISCUSSION

The calcium and vitamin D fortified milk was developed and its nutrition absorption analysis was carried out using *in-vitro* models based on human physiology (**Sachdeva *et al.*, 2015b**) although their potential predictive value regarding absorption in humans should be validated in different *in-vivo* situations **(Oomen 2003)**.

Modifications in the *in vitro* method

Compositions and concentrations of inorganic and organic solutions, gastric and duodenal juices and bile constituents were carefully duplicated as described by **Granado-Lorencio *et al.*, (2007)**. He determined recovery of nutrients in the supernatant after decantation for 16 h at room temperature and after low speed centrifugation (3200 g for 20 min). However, numerous other methods have discussed the model where diffusion used to determine bioavailability **(Miller *et al.*, 1981; Drago and Valencia 2004; Etcheverry *et al.*, 2012)**. In human digestion, nutrients in digested food passed into the blood vessels in the wall of the intestine through the process of diffusion. Certain dietary factors can influence calcium solubility, thereby affecting calcium bioavailability at the absorptive surface of intestine cell. Thus, *in-vitro* methods might be useful in comparing the bioavailability of different calcium salts that are contained in dietary supplements or when added as food fortificants **(Etcheverry *et al.*, 2012)**. Therefore, dialysis of digested milk carried out using cellulose dialysis membranes (molecular weight cut-off, 12000-14000 Da). The nutrients were permeated through dialysis membrane were considered as absorbed. The absorption of added nutrients calculated using AAS for calcium and HPLC for vitamin D.

Absorption of added nutrients

Absorption of calcium from fortified milk

The calcium absorption from fortified milk was determined and values ranges between 40.43 to 53.08 % for calcium phosphate fortified groups and 40.43 to 47.90% for calcium citrate fortified group (Table 1). **Buchowski (2016)** reported similar calcium absorption range with 30-50% from dairy foods. The calcium absorption in calcium fortified group and vitamin D fortified group was statistically ($p>0.05$) similar from both calcium salts, however, in calcium and vitamin D_2 fortified samples; calcium phosphate showed higher ($p<0.05$) calcium absorption than calcium citrate. **Malyugina (2020)** carried out a systematic review and concluded that vitamin D_2 increases the calcium absorption.

Absorption of vitamin D from fortified milk

The vitamin D fortified at the rate of 600IU/L of milk, absorption was determined using dialysis tubing membrane, and results presented in Table 2. In control sample, vitamin D not detected. The vitamin D absorption ranges between 397.08 to 484.08 IU/L for vitamin D fortified milk and calcium (600mg/L) and vitamin D fortified milk in calcium phosphate and vitamin D fortified samples. In calcium citrate and vitamin D fortified samples, vitamin D absorption ranged between 406.02 to 478.56 IU/L for vitamin D fortified milk and calcium (600mg/L) and vitamin D fortified milk in calcium citrate and vitamin D fortified samples. Results showed that vitamin D absorption increased when fortification with calcium and its absorption increased with increase in calcium content. Vitamin D absorption found similar ($p>0.05$) for both calcium salts in all different groups, respectively. Fortified milk is the source of extra essential nutrients and help in fulfilling RDA; however, it has health benefits also. **Morvoridzadeh (2020)** carried out meta-analysis and reported that calcium and vitamin D co supplementation significantly reduce diastolic blood pressure; however, non-significant difference observed in systolic blood pressure. **Mandlik (2020)** supplemented vitamin D and calcium to children and reported improvement in bone health parameters and increase PTH level.

Table 1 Bioavailability of calcium from fortified milk

Samples	Total Calcium Content	Bioavailability of calcium (%)	
		Calcium Phosphate	Calcium Citrate
Control	1500 mg/L	43.27±0.37[a]	42.36+0.21[a]
Calcium 500 mg/L	2000 mg/L	40.54±0.10[a]	40.54+0.29[a]
Calcium 600 mg/L	2100 mg/L	40.43±0.21[a]	40.43 +0.26[a]
Vitamin D 600 IU/L	1500 mg/L	48.96±3.79[b]	49.29+0.64[b]
Calcium 500 mg/L + Vitamin D 600 IU/L	2000 mg/L	50.14±3.29[b]	46.31+0.12[b]
Calcium 600 mg/L + Vitamin D 600 IU/L	2100 mg/L	53.08±2.88[b]	47.90+0.41[b]

Values presented in Table are means±SEM (n=3).
[a-b]Samples represented with different letters are significantly different (P<0.05) from each other in rows.

Table 2 Absorption of vitamin D different milk samples

Samples	Absorption of vitamin D	
	Calcium phosphate + Vitamin D_2	Calcium citrate + Vitamin D_2
Control	Not detected	Not detected
Vitamin D 600 IU/L	397.08±8.42[a]	406.02±5.01[a]
Calcium 500 mg/L + Vitamin D 600 IU/L	454.92±6.92[b]	452.34±7.04[b]
Calcium 600 mg/L + Vitamin D 600 IU/L	484.08±7.88[c]	478.56±5.20[c]

Values presented in Table are means±SEM (n=3).
[a-b]Samples represented with different letters are significantly different (P<0.05) from each other in rows.

Weight of rats

The rats divided into four groups and of similar weight at zero day. After every seven days upto 28 days, significant (P<0.05) increase in weight was observed in all rat groups, respectively. In between groups, Control and vitamin D fortified feed groups showed statistical similar weight; however, calcium fortified group showed significantly higher weight than vitamin D and control groups. The both nutrients fortified group showed highest weight gain in comparison to all other groups (Table 3). From weight trends, it was observed that calcium and vitamin D fortification has positive effect on growth of growing rats.

Table 3 Weight change in rats during 28 days study

Groups	Weight of rats				
	0 day	After 7 days	After 14 days	After 21 days	After 28 days
Control	54.26±1.63aA	91.61±4.27aB	126.37±6.41aC	164.97±7.52aD	190.06±8.13aE
Vitamin D group	53.39±1.72aA	86.71±4.21aB	122.69±3.83aC	158.00±3.60aD	186.20±3.24aE
Calcium Phosphate group	54.06±1.59aA	98.33±4.02bB	141.02±6.21bC	180.49±6.78bD	208.02±7.57bE
Calcium phosphate and Vitamin D group	53.82±1.64aA	101.49±2.36bB	153.52±3.37bC	197.85±4.40bD	226.38±4.91bE

Values presented in Table are means±SEM (n=3).
[a-b]Samples represented with different letters are significantly different (P<0.05) from each other in column.
[A-B]Samples represented with different letters are significantly different (P<0.05) from each other in rows.

Effect of fortification on nitrogen utilization indices

The nitrogen utilization indices of fortified milk samples determined *in-vivo* in rats. The results obtained presented below

Protein efficiency ratio (PER)

As per **FAO (2011)**, food having PER more than 2.5 (Standard casein) is consider as good source of protein. The PER of milk is 3.09. The PER of our samples was 4.65 to 4.99 which is far higher than standard casein. It was observed that fortification increased PER. The PER of combine fortified (calcium and vitamin D) group was significantly higher than single fortified groups. Vitamin D fortified group showed significantly higher PER than calcium-fortified group.

Digestibility coefficient (DC)

Maathuis (2017) reported that milk DC ranged between 65-75%. In present study, the DC ranged between 66.52 to 76.27% for calcium-fortified group, calcium and vitamin D fortified group, respectively. It was observed that vitamin D fortification increased DC, whereas calcium fortification has no effect on DC. When both nutrients fortified in combination significant higher DC observed.

Biological value (BV)

The BV is the most important indices for nitrogen utilization. Egg is standard protein and considered as 100% BV. Food products with more than 95% BV considered as good source of protein. The BV in present study ranged between

95.82-96.69% for control and vitamin D and calcium fortified group, respectively. Both nutrient fortified group showed significantly higher ($p<0.05$) BV than all other three groups (Figure 1).

Net protein utilization (NPU)

The NPU is the finally retain portion of protein from consumed food. This protein finally converted into muscles and other protein metabolites. The NPU in present study ranged between 63.81 to 73.74% for control and calcium and vitamin D fortified groups, respectively. Both nutrient fortified group showed significantly higher NPU than vitamin D fortified group. Vitamin D fortified group showed higher NPU than calcium and control group. From above result, it was observed that vitamin D increases the retention of protein in body. It might be due to role of vitamin in function of kidney.

As such, no study published which can show direct interaction of vitamin D and calcium on protein digestion. Some studies are there from which some connection of these nutrient with protein observed.

Skeletal muscles expresses vitamin D receptor and may require vitamin D for maximizing its function **(Boland 2011)**. Vitamin D deficiency impairs proximal muscle function and is through to pre-dispose falls in elderly. An improvement in muscle strength and movement was recorded when vitamin D level rose from 4 to 16ng/ml and continued to improve upto 40ng/ml **(Bischoff-Ferrari *et al.*, 2006)**. From literature, it is observed that 800IU/day vitamin D supplementation with calcium can prevent risk of falls. Vitamin D receptor and membrane associated rapid response steroid binding protein found in the cell membrane bind $1,25(OH)_2D$ and initiate the activation of numerous pathways involving various protein kinase **(Olmos-Ortiz *et al.*, 2015)**.

Vitamin D enhances the osteocalcin (non-collagenous protein) expression in bones. Vitamin D with PTH enhances bone resorption by stimulating the osteoblast to express receptor activator of nuclear factor Kappa β Ligand on cell membrane as well as releasing it into the circulation **(Charoenngam *et al.*, 2019)**. **Takiar (2015)** carried out research on effect of vitamin D binding protein, VDD and fracture risk. He reported that vitamin D were associated with higher incidence of hospitalized fractures. **Ping-Delfos (2011)** reported that higher calcium and vitamin D diet showed higher diet induced thermogenesis and fat oxidation. It also reduces carbohydrate oxidation rates. From above results, it can be suggested that high calcium and vitamin D enhance metabolism but reduce carbohydrates oxidation, therefore, to maintain hemostasis body will absorb more protein. Due to all these effects associated with vitamin D and calcium, there is possibility to have positive effect on nitrogen utilization or protein digestibility.

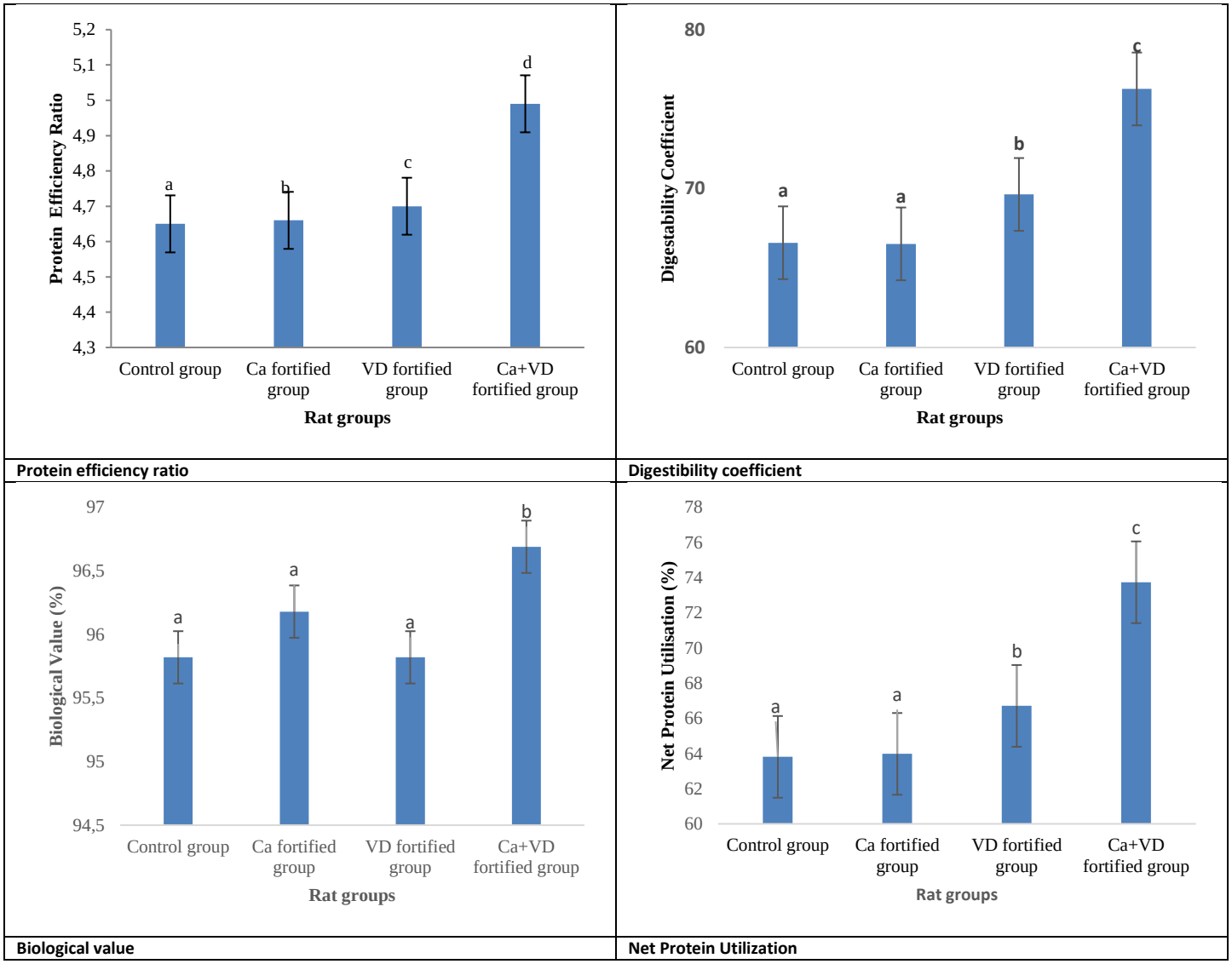

Figure 1 Nitrogen utilization indices of fortified groups. [a-b]Samples represented with different letters are significantly different ($P<0.05$) from each other. Error bars show the variations of three determinations in terms of standard error of mean.

CONCLUSION

A simple *in-vitro* method (simulated gastrointestinal tube) was used to determine the bioavailability of added nutrients from fortified milk. It is fact that vitamin D enhances calcium absorption; however, in present study, obtained results showed that both nutrients had synergistic effect. Nitrogen absorption and utilization indices showed that both nutrient fortification had positive effect on protein utilization. There is requirement of clinical/human trials to prove synergistic effect of vitamin D and calcium on protein utilization indices.

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REFERENCES

Paul, S., Kaushik, R., Chawla, P., Uppadhyay, S., Rawat, D., & Akhtar, A. (2024). Vitamin-D as a multifunctional molecule for overall well-1 being: An Integrative

review. *Clinical Nutrition ESPEN*, 62, 10-21.https://doi.org/10.1016/j.clnesp.2024.04.016.
Kaushik, R., Sachdeva, B., Arora, S., Kapila, S., & Wadhwa, B. K. (2014a). Bioavailability of vitamin D2 and calcium from fortified milk. *Food Chemistry*, 147(2014), 317-321.http://dx.doi.org/10.1016/j.foodchem.2013.09.150
Boland, R. L. (2011). VDR activation of intracellular signaling pathways in skeletal muscle. *Molecular Cell Endocrinology* 347, 11–16.https://doi.org/10.1016/j.mce.2011.05.021
Bischoff-Ferrari, H. A., Giovannucci, E., Willett, W. C., *et al.*, (2006). Estimation of optimal serum concentrations of 25- hydroxyvitamin D for multiple health outcomes. *American Journal of Clinical Nutrition*, 84, 18–28.https://doi.org/10.1093/ajcn/84.1.18
Kiely, M., Hemmingway, A. & O'Callaghan, K. M. (2017). Vitamin D in pregnancy: current perspectives and future directions. *Therapeutic Advances in Musculoskeletion Disorder*, 9(6), 145-154.https://doi.org/10.1177/1759720X17706453
Olmos-Ortiz, A., Avila, E., Durand-Carbajal, M., & Díaz, L. (2015). Regulation of calcitriol biosynthesis and activity: focus on gestational vitamin D deficiency and adverse pregnancy outcomes. *Nutrients*, *7*(1), 443–480.https://doi.org/10.3390/nu7010443
Chen, N. C., Hsu, C. Y., Mao, P. C., Dreyer, G., Wu, F. Z., & Chen, C. L. (2020). The effects of correction of vitamin D deficiency on arterial stiffness: A systematic review and updated meta-analysis of randomized controlled trials. *The Journal of steroid Biochemistry and Molecular Biology*, *198*, 105561.https://doi.org/10.1016/j.jsbmb.2019.105561
Holt, R., Juel Mortensen, L., Harpelunde Poulsen, K., Nielsen, J. E., Frederiksen, H., Jørgensen, N., Jørgensen, A., Juul, A., & Blomberg Jensen, M. (2020). Vitamin D and sex steroid production in men with normal or impaired Leydig cell function. *The Journal of Steroid Biochemistry and Molecular Biology*, *199*, 105589.https://doi.org/10.1016/j.jsbmb.2020.105589
Zheng, S., Yang, J., Hu, X., Li, M., Wang, Q., Dancer, R., Parekh, D., Gao-Smith, F., Thickett, D. R., & Jin, S. (2020). Vitamin D attenuates lung injury via stimulating epithelial repair, reducing epithelial cell apoptosis and inhibits TGF-β induced epithelial to mesenchymal transition. *Biochemical Pharmacology*, *177*, 113955.https://doi.org/10.1016/j.bcp.2020.113955
Colak R, Anil M, Yasar F, Bakiler, R., Pirgon, O., Helyaci, M., Dundar, B. (2020). Metabolic disturbances and cardiovascular risk factors in obese children with vitamin D deficiency. *Archives de Pediatrie : Organe Officiel de la Societe Francaise de Pediatrie*. 27(3), 140-145.https://doi.org/10.1016/j.arcped.2019.12.005
Kilpatrick, L.E., Boggs, A. S. P., Davis, W. C., Long, S. E., Yen, J. H., & Phinney, K. W. (2020). Assessing a method and reference material for quantification of vitamin D binding protein during pregnancy. *Clinical Mass Spectrometry*, 16, 11-17.https://doi.org/10.1016/j.clinms.2020.01.002
Sempos, C. T., Heijboer, A. C., Bikle, D. D., Bollerslev, J., Bouillon, R., Brannon, P. M., DeLuca, H. F., Jones, G., Munns, C. F., Bilezikian, J. P., Giustina, A., & Binkley, N. (2018). Vitamin D assays and the definition of hypovitaminosis D: results from the First International Conference on Controversies in Vitamin D. *British Journal of Clinical Pharmacology*, *84*(10), 2194–2207.https://doi.org/10.1111/bcp.13652
Sun, X., Acquah, C., Aluko, R., & Udenigwe, C. (2020). Considering food matrix and gastrointestinal effects in enhancing bioactive peptide absorption and bioavailability. *Journal of Functional Foods*, *64*, 103680. https://doi.org/10.1016/j.jff.2019.103680
Charoenngam, N., Shirvani, A., & Holick, M. F. (2019). Vitamin D for skeletal and non-skeletal health: What we should know. *Journal of Clinical Orthopaedics and Trauma*, *10*(6), 1082–1093.https://doi.org/10.1016/j.jcot.2019.07.004
Cashman, K. D. and O'Dea, R. (2019). Exploration of strategic food vehicles for vitamin D fortification in low/lower middle income countries. *The Journal of Steroid Biochemistry and Molecular Biology*, 195, 105479.https://doi.org/10.1016/j.jsbmb.2019.105479
Kaushik, R. & Arora, S. (2017). Effect of calcium and vitamin D_2 fortification on physical, microbial, rheological and sensory characteristics of yoghurt. *International Food Research Journal*, 24(4), 1744-1752.http://www.ifrj.upm.edu.my/24%20(04)%202017/(52).pdf
Kaushik, R. & Sachdeva, B. & Arora, S. (2017b). Effect of calcium and vitamin D2 fortification on quality characteristics of dahi. *International Journal of Dairy Technology*, 70(2), 269-276.https://doi.org/10.1111/1471-0307.12333
Rana, B., Kaushik, R., Kaushal, K., Arora, S., Kaushal, A., Gupta S., Upadhay, N., Rani, P. & Sharma P. (2018). Physicochemical and electrochemical properties of zinc fortified milk. *Food Bioscience*, 21, 117-124.https://doi.org/10.1016/j.fbio.2017.12.008
Singh, G., Arora, S., Sharma, G. S., Sindhu, J. S., Kansal, V. K., & Sangwan, R. B. (2007). Heat stability and calcium bioavailability of calcium fortified milk. *LWT - Food Science Technology*, 40(4), 625-631.https://doi.org/10.1016/j.lwt.2006.03.009
Ross, A. C., Taylor, C. L., Yaktine, A. L., *et al.*, (2011). Dietary Reference Intakes for Calcium and Vitamin D. Washington (DC): National Academies Press (US); 2011. 3, Overview of Vitamin D.https://www.ncbi.nlm.nih.gov/books/NBK56070/
Laird, E., Ward, M., McSorley, E., Strain, J. J. & Wallace, J. (2010). Vitamin D and Bone Health; Potential Mechanisms. *Nutrients*, 2, 693-724.https://doi.org/10.3390/nu2070693
Sachdeva, B., Kaushik, R. & Arora, S. (2015a). Bioavailability of iron in multiple fortified milk. *Journal of Food Science and Technology*, 52(9), 6017–6023.https://doi.org/10.1007/s13197-015-1711-9
Sachdeva, B., Kaushik, R. & Arora, S. (2015b). Impact of fortification with iron salts and vitamin A on the physico-chemical properties of laboratory pasteurized toned milk and bioaccessibility of the added nutrients. *International Journal of Dairy Technology*, 68(2), 253-260.https://doi.org/10.1111/1471-0307.12185
Kaushik, R., Sachdeva, B., Arora, S. & Gupta, C. (2015a). Effect of fat content on sensory and physico-chemical properties of calcium and vitamin D fortified milk. *International Journal of Dairy Technology*, 68(1), 135-143.https://doi.org/10.1111/1471-0307.12166
Kaushik, R., Sachdeva, B., & Arora, S. (2015b). Heat stability and thermal properties of calcium fortified milk. *Cyta-Journal of Food*, *13*(2), 305-311.https://doi.org/10.1080/19476337.2014.971346
Kaushik, R., & Arora, S. (2023). Standardization of Atomic Absorption Spectrometry protocol for calcium analysis in milk and milk products. *Journal of Food Chemistry and Nanotechnology*, 9(1), 36-42.https://doi.org/10.17756/jfcn.2023-148
Kaushik, R., Sachdeve, B., Arora, S., Wadhwa, B.K. (2014b). Development of an analytical protocol for the estimation of vitamin D_2 in fortified toned milk. *Food Chemistry*, 154(2014), 567-573.https://doi.org/10.1016/j.foodchem.2013.11.085
AOAC (2023). Official methods of analysis. The association of official analytical chemists. 18th edition. 481. North Fredrick Avenue Gaithersburg, Maryland, USA.https://www.aoac.org/official-methods-of-analysis/
Chawla, P., Najda, A., Bains, A., Nurzy´nska-Wierdak, R., Kaushik, R., & Tosif, M.M. (2021) Potential of Gum Arabic Functionalized Iron Hydroxide Nanoparticles Embedded Cellulose Paper for Packaging of Paneer. *Nanomaterials*, 11, 1308. https://doi.org/10.3390/nano11051308
Oomen, A. G., Tompelberg, C. J. M., Bruil, M. A., Dobbe, C. J. G., Pereboom, D. P. K.H., & Slips, A. J. A. M. (2003). Development of an *in vitro* digestion model for estimating the bioavailability of soil contaminants. *Archives of Environmental Contamination and Toxicology*, 44(3), 281-287. https://doi.org/10.1007/s00244-002-1278-0
Granado-Lorencio, F., Olmedilla-Alonso, B., Herrero-Barbudo, C., Blanco-Navarro, I., Perez-Sacristan, B., & Blazquez-Garcıa, S. (2007). *In vitro* bioavailability of carotenoids and tocopherols from fruits and vegetables. *Food Chemistry*, 102, 641-648.https://doi.org/10.1177/153537020623101110
Miller, D. D., Schricker, B. R., Rasmussen, R. R. & Van Campen, D. (1981). An *in vitro* method for estimation of iron availability from meals. *American Journal of Clinical Nutrition*, 34, 2248–2256.https://doi.org/10.1093/ajcn/34.10.2248
Drago, S. R., & Valencia, M. E. (2004). Influence of components of infant formulation *in vitro* iron, zinc, and calcium availability. *Journal of Agricultural Food Chemistry*, 52, 3202–3207.https://doi.org/10.1021/jf035191e
Etcheverry, P., Grusak, M. A. & Fleige, L. E. (2012). Application of *in vitro* bioavailability and bioavailability methods for calcium, carotenoids, folate, iron, magnesium, polyphenols, zinc, and vitamins B_6, B_{12}, D, and E. *Frontiers of Physiology*, 3, 1-22.https://doi.org/10.3389/fphys.2012.00317
Buchowski, M.S. (2016). Calcium in the Context of Dietary Sources and Metabolism. CH-1. Food and Nutritional Components in Focus No. 10 Calcium: Chemistry, Analysis, Function and Effects Edited by Victor R. The Royal Society of Chemistry. https://doi.org/10.1039/9781782622130-00003
Malyugina, O.A. and Markov, A.A. (2020). The practicability of the application of vitamin D in combination with vitamin K for the improvement of bone tissue metabolism. *Systematic Reviews in Pharmacy*, 11(6), 445-448.https://doi.org/10.1155/2017/7454376
Morvaridzadeh, M., Sepidarkish, M., Fazelian, S., Rahimlou, M., Omidi, A., Ardehali, S. H., Sanoobar, M., & Heshmati, J. (2020). Effect of Calcium and Vitamin D Co-supplementation on Blood Pressure: A Systematic Review and Meta-Analysis. *Clinical therapeutics*, *42*(3), e45–e63.https://doi.org/10.1016/j.clinthera.2020.01.005
Mandlik, R., Mughal, Z., Khadilkar, A., Chiplonkar, S., Ekbote, V., Kajale, N., Patwardhan, V., Padidela, R. & Khadilkar, V. (2020). Occurrence of infections in schoolchildren subsequent to supplementation with vitamin D-calcium or zinc: a randomized, double-blind, placebo-controlled trial. *Nutrition Research and Practice*, *14*(2), 117–126. https://doi.org/10.4162/nrp.2020.14.2.117
FAO (2011). Expert consultation. Dietary protein quality evaluation in human nutrition. FAO Food Nutrition Paper. 92, 1–66. https://www.fao.org/ag/humannutrition/35978-02317b979a686a57aa4593304ffc17f06.pdf
Maathuis, A., Hayenaar, R., He, Y.T. & Bellmann, S. (2017). Protein digestion and quality of goat and cow milk infant formula and human milk under simulated infant conditions. *JPGN*, 65(6), 661–666.https://doi.org/10.1097/MPG.0000000000001740
Takiar, R., Lutsey, P. L., Zhao, D., Guallar, E., Schneider, A. L. C., Grams, M. E., Appel, L. J., Selvin, E. & Michos, E. D. (2015). The associations of 25-hydroxyvitamin D levels, vitamin D binding protein gene polymorphisms, and race with risk of incident fracture-related hospitalization: Twenty-year follow-up in a

bi-ethnic cohort (the ARIC Study). *Bone*, 78, 94-101.https://doi.org/10.1016/j.bone.2015.04.029
Ping-Delfos, W. C. S., & Soares, M. (2011). Diet induced thermogenesis, fat oxidation and food intake following sequential meals: Influence of calcium and vitamin D. *Clinical Nutrition*, 30, 376-383.https://doi.org/10.1016/j.clnu.2010.11.006