2.5. Digestible Indispensable Amino Acid Score (DIAAS)

DIAAS was calculated as recommended by the FAO/WHO using the following equation:

DIAAS% = 100 × [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein)/(mg of the same dietary indispensable amino acid in 1 g of the reference protein)] (FAO/WHO, 2013). Ideally, the ileal amino acid digestibility should be used for the calculation of PDCAAS, however the use of fecal digestibility is considered acceptable until such time as a dataset of true ileal digestibility is developed (FAO/WHO, 2013).

2.6. In vitro Protein Digestibility-Corrected Amino Acid Score (In vitro PDCAAS)

An *in vitro* digestibility assay was also performed on each sample as previously described (Nosworthy, Franczyk et al., 2017). Briefly, 62.5 mg of protein was incubated at 37 °C for ten minutes with a protease cocktail containing trypsin, chymotrypsin and protease in duplicate. The resulting pH drop was recorded for 10 min and the *in vitro* protein digestibility was calculated as follows (where the $\Delta pH_{10\ min}$ is the change in pH in 10 min from the initial pH of approximately 8.0)

$$IVDP\% = 65.66 + 18.10 \cdot \Delta pH_{10min}$$

The *In Vitro* PDCAAS was calculated as a product of the amino acid score and IVPD%.

2.7. Protein Efficiency Ratio (PER)

As mandated by Health Canada, PER is determined over a 28 day growth period for rats consuming feed *ad libitum* (Health Canada, 1981). The PER was calculated as weight gain (g) divided by the amount (g) of protein consumed over 28 days. Values were also adjusted to a standardized 2.5 PER value for the reference casein (analyzed concurrently).

2.8. Statistics

Results were compared via two-way ANOVA with post hoc analysis using Tukey's multiple comparison test, while the relationship between *in vivo* and *in vitro* digestibilities and corrected amino acid scores was determined via regression analysis (GraphPad Prism, 7.0).

3. Results and discussion

3.1. Proximate analysis

The dry matter, crude fat and crude protein for untreated and processed lentil flours are found in Table 1 on an 'as is' basis. Untreated red and green lentils had similar dry matter percentage (92.12% vs 91.38%) which was representative of previous work (Canadian Nutrient File, 2016). Processed red and green lentil flours also showed similar dry matter with the extrudates (95.41% vs 95.13%), cooked samples (99.57% vs 99.47%) and baked samples (97.49% vs 97.32%) being separated by less than 0.3%. The higher percentage of dry matter of the cooked samples was a result of an additional freeze drying operation performed post-boiling. Untreated red lentils had higher fat content than green lentils (1.78% vs 1.13%) and both were greater than the previously reported value of approximately 1% (Canadian Nutrient File, 2016; Wang & Daun, 2006). The protein content of the untreated red and green lentils was 25.13% and 23.93% respectively and was similar to previously reported results of 25–26% (Canadian Nutrient File, 2016; Wang et al., 2009). Processing increased protein content for both red and green lentils with a range of 24.65% for extruded green lentil to 26.86% for extruded red lentils, however in all cases the increase in protein content was less than 2%. These results indicate that processing via extrusion, baking or cooking did not dramatically alter the protein content of these samples.

3.2. Amino acid scores and true protein digestibility

The amino acid composition for all ingredients is presented in Table 1 with the resulting amino acid scores presented in Table 2. The first limiting amino acid score for both red and green lentils was the sulfur amino acids, methionine and cysteine, with values ranging from 0.57, baked green lentil, to 0.68, extruded red lentil. Tryptophan was also limiting in the processed lentil samples with a range of 0.69, extruded red lentil, to 0.78, extruded green lentils. Lentils have been previously described as limiting in sulfur amino acids and tryptophan (Nosworthy, Neufeld et al., 2017; Sarwar & Peace, 1986). After processing, both lentil varieties had the highest amino acid score after extrusion (0.68 and 0.66 for red and green lentils respectively), followed by cooking (0.63 and 0.61) and then baking (0.66 and 0.57). Another study investigating the effect of cooking on amino acid composition determined an amino acid score of 0.59 for cooked red lentils and 0.71 for cooked green lentils (Nosworthy, Neufeld et al., 2017). While these values differ from the current study (0.59 vs 0.63 and 0.71 vs 0.61 for red and green lentils respectively) this may be due to the use of different varieties or crop year as those conditions can alter amino acid composition.

The *in vivo* and *in vitro* protein digestibility values are found in Table 3. The true protein digestibility values of processed red lentil flour was comparable between extruded (92.38%), cooked (90.95%) and baked (88.80%), with processed green lentil flour having a similar relationship (extruded 86.02%, cooked 86.42%, baked 83.05%). Overall, green lentil flour had lower digestibility compared to red lentil flour. These results are similar to previous studies where cooked lentils had true protein digestibilities of 90.60% for red lentils and 87.89% for green lentils compared to this study which found 90.95% and 86.42% respectively (Nosworthy,

Table 1
Proximate analysis and amino acid composition of untreated, extruded, cooked and baked red and green lentil flour presented on an as-is basis.

	%DM[a]	%CF[c]	%CP[b]	ASP	THR	SER	GLU	PRO	GLY	ALA	CYS	VAL	MET	ILE	LEU	TYR	PHE	HIS	LYS	ARG	TRP
Casein	93.56	0.20	86.48	7.78	3.35	5.64	20.05	9.77	1.35	3.16	0.78	5.02	1.45	3.84	8.39	4.83	4.59	2.74	6.96	3.12	1.08
Red Lentil																					
Untreated	92.12	1.78	25.13	2.69	0.78	1.21	3.55	0.60	0.80	0.98	0.22	0.97	0.206	0.77	1.67	0.63	1.09	0.60	1.43	1.88	0.20
Extruded	95.41	1.08	26.86	3.30	0.96	1.47	4.38	0.79	0.97	1.25	0.24	1.18	0.22	0.96	1.88	0.68	1.33	0.79	1.81	2.01	0.20
Cooked	99.57	1.62	26.62	3.31	0.96	1.56	4.41	0.91	0.85	1.23	0.22	1.21	0.21	1.03	2.19	0.71	1.43	0.77	1.83	2.28	0.22
Baked	97.49	2.34	25.93	3.25	1.00	1.54	4.47	0.96	1.00	1.29	0.20	1.13	0.19	1.00	2.05	0.67	1.26	0.80	1.57	2.30	0.20
Green Lentil																					
Untreated	91.38	1.13	23.93	2.81	0.83	1.31	3.86	0.90	0.87	1.15	0.20	1.02	0.19	0.89	1.75	0.67	1.17	0.66	1.61	2.22	0.18
Extruded	95.13	1.48	24.65	3.05	0.89	1.36	4.02	0.73	0.91	1.20	0.21	0.99	0.20	1.02	1.93	0.64	1.24	0.73	1.71	2.14	0.21
Cooked	99.47	2.06	25.67	3.07	0.91	1.45	4.00	0.82	0.90	1.20	0.20	1.19	0.20	1.00	2.05	0.67	1.32	0.71	1.76	2.11	0.21
Baked	97.32	2.21	25.44	2.53	0.79	1.24	3.67	0.53	0.88	1.14	0.18	1.13	0.18	0.89	1.69	0.57	1.06	0.67	1.37	1.88	0.20

[a] DM = dry matter content.
[b] CF = crude fat determined by hexane extraction.
[c] CP = crude protein = nitrogen content (determined by Dumas analysis) × 6.25.

Table 2
Amino Acid Score of extruded, cooked and baked red and green lentil flour.

	THR	VAL	MET + CYS	ILE	LEU	PHE + TYR	HIS	LYS	TRP
Casein	1.14	1.66	**1.03**	1.59	1.47	1.73	1.67	1.39	1.13
Red Lentil									
Extruded	1.05	1.25	**0.68**	1.28	1.06	1.19	1.55	1.16	0.69
Cooked	1.06	1.30	**0.63**	1.38	1.25	1.28	1.53	1.19	0.75
Baked	1.14	1.25	**0.61**	1.37	1.20	1.18	1.63	1.04	0.71
Green Lentil									
Extruded	1.06	1.14	**0.66**	1.48	1.19	1.21	1.55	1.20	0.78
Cooked	1.05	1.33	**0.61**	1.39	1.21	1.23	1.46	1.18	0.74
Baked	0.92	1.27	**0.57**	1.25	1.00	1.02	1.38	0.93	0.72

Bolded values indicate the first limiting amino acid. The reference pattern used to calculate the amino acid scores was as followed (mg/g protein): Thr-34, Val-35, Met + Cys-25, Ile-28, Leu-66, Phe + Tyr-63, His-19, Lys-58, Trp-11.

Neufeld et al., 2017). Autoclaved pulse flour, which has been used as a surrogate for a heat exposed baking process, found lentil digestibility to be between 81.4–85% while this study determined the true protein digestibility of baked flours to be 88.80% for red lentils and 83.05% for green lentils (FAO/WHO, 1991; Porres et al., 2002). Similar results were determined using an *in vitro* method for determination of protein digestibility of red lentil ranging from 85.03% for baked flours to 88.01% for extruded flour and 79.33% in baked green lentils to 84.30% for extruded flour. While the overall pattern is similar between the *in vivo* and *in vitro* measurements, it is worth noting that in all cases the *in vitro* measurement of protein digestibility was lower than that determined by the standard *in vivo* rodent bioassay.Table 4

3.3. In vivo and in vitro protein digestibility corrected amino acid scores

The protein digestibility corrected amino acid score (PDCAAS) is presented in Table 3. This measurement of protein quality is influenced by both the amino acid score and protein digestibility (FAO/WHO, 1991). The PDCAAS values ranged from a low of 47.14% (baked green lentils) to 63.01% (extruded red lentils). In both classes, extruded flour had the highest PDCAAS (63.01% for red lentils, 57.09% for green lentils) followed by cooked (57.40% red, 53.93% green) and baked (53.84% red, 47.14% green). In all cases, processed red lentil flour had higher PDCAAS values due to the combination of a higher amino acid score and greater protein digestibility. Previously, the PDCAAS of cooked red lentils has been found to be 53.8% while green lentils were 62.8% (Nosworthy, Neufeld et al., 2017). A study investigating the effect of autoclaving on the PDCAAS of lentils determined a value of 66.4%, primarily due to a higher amino acid score (0.82) compared to that found in this study (average of 0.59 between red and green lentils) (Porres et al., 2002). In this study the *In Vitro* PDCAAS values were consistently lower than PDCAAS with a range of 1.14% (extruded green lentil) to 3.97% (cooked red lentil), however the general pattern of extruded flours having the highest value, and baked flours having the lowest, was maintained.

The current requirements for protein quality determination mandate the use of an *in vivo* bioassay, but a reduction in animal utilization for regulatory purposes is desirable (FAO/WHO, 1991). In order to determine whether an *in vitro* method of protein digestibility would relate to that found *in vivo*, a one-step pH drop method was utilized (Hsu, Vavak, & Satterlee, 1977; Tinus, Damour, Van Riel, & Sopade, 2012; Nosworthy, Neufeld et al., 2017). The relationship between *in vivo* and *in vitro* protein digestibility and measurement of protein quality was determined via correlation analysis and is presented in Fig. 1. A strong correlation was found between *in vitro* protein digestibility and true protein digestibility ($R^2 = 0.8934$). As both methods are using the same protein ingredient, the amino acid score remains the same whether calculating *In Vitro* PDCAAS or PDCAAS, the correlation between these values is stronger than digestibility alone ($R^2 = 0.9971$ for PDCAAS vs *In Vitro* PDCAAS; vs $R^2 = 0.8934$ for IVPD vs TPD). When testing the possibility of whether casein is skewing the correlation analysis, the correlation remains significantly high ($R = 0.9631$) if that data point is removed. These results are similar to previous comparisons between *in vivo* and *in vitro* methods of determining protein quality with $R^2 = 0.9898$ and $R^2 = 0.9280$ (Nosworthy & House, 2017; Nosworthy, Neufeld et al., 2017). Another study compared multiple methods for determining the *in vitro* PDCAAS of chickpea protein fractions to *in* vivo data and found similar results for the method used in the present study ($R^2 = 0.9442$) (Tavano, Neves, & da Silva Junior, 2016). These strong correlations suggest that an *in vitro* method for determining protein quality (*In Vitro* PDCAAS) could be used as a surrogate for *in vivo* evaluation of pulse protein ingredients but a larger dataset is required for further confirmation.

Table 3
Adjusted Protein Efficiency Ratio, Protein Digestibility-Corrected Amino Acid Scores and *In Vitro* Protein Digestibility-Corrected Amino Acid Scores of extruded, cooked and baked red and green lentil flour.

	Adj. Per	AAS[a]	%TPD[b]	IVPD[c]	PDCAAS[d]	*In Vitro* PDCAAS[e]
Casein	2.50	1.03	96.11 (1.4)	91.36 (0.7)	99.09	94.19
Red Lentil						
Extruded	1.05	0.68	92.38 (2.3)	88.01 (2.8)	63.01	60.03
Cooked	1.14	0.63	90.95 (2.2)	84.67 (1.1)	57.40	53.43
Baked	0.79	0.61	88.80 (2.4)	85.03 (0.5)	53.84	51.55
Green Lentil						
Extruded	1.08	0.66	86.02 (2.0)	84.30 (0.0)	57.09	55.95
Cooked	0.98	0.61	86.42 (2.0)	84.03 (1.2)	52.92	51.46
Baked	0.88	0.57	83.05 (1.9)	79.33 (1.0)	47.14	45.03

[a] AAS = amino acid score.
[b] %TPD = % true protein digestibility.
[c] IVPD = *in vitro* protein digestibility.
[d] PDCAAS = protein digestibility corrected amino acid score.
[e] *In Vitro* PDCAAS = *in vitro* protein digestibility corrected amino acid score. n = 10 for Adj. PER and %TPD; n = 2 for IVPD and n = 1 for AAS, PDCAAS, *In Vitro* PDCAAS. Numbers in parentheses indicate SD where applicable. PDCAAS is calculated as the product of AAS and %TPD while *In Vitro* PDCAAS is the product of AAS and IVPD.

Table 4
Digestible Indispensable Amino Acid values of extruded, cooked and baked red and green lentil flour.

	THR	VAL	MET + CYS	ILE	LEU	PHE + TYR	HIS	LYS	TRP	DIAAS[a]
Casein	1.20	1.30	**0.92**	1.34	1.41	2.01	1.52	1.36	1.41	0.92
Red Lentil										
Extruded	1.06	0.94	**0.58**	1.03	0.98	1.33	1.36	1.09	0.83	0.58
Cooked	1.06	0.96	**0.53**	1.10	1.14	1.41	1.32	1.10	0.88	0.53
Baked	1.11	0.90	**0.50**	1.07	1.06	1.27	1.38	0.94	0.82	0.50
Green Lentil										
Extruded	1.00	0.80	**0.53**	1.11	1.02	1.26	1.27	1.05	0.87	0.53
Cooked	0.99	0.93	**0.49**	1.05	1.04	1.29	1.20	1.04	0.82	0.49
Baked	0.84	0.86	**0.44**	0.91	0.83	1.03	1.09	0.79	0.77	0.44

Notes: DIAAS was calculated using true protein digestibility. Bolded values reflect first limiting amino acid. [a]DIAAS = Digestible Indispensable Amino Acid Score.

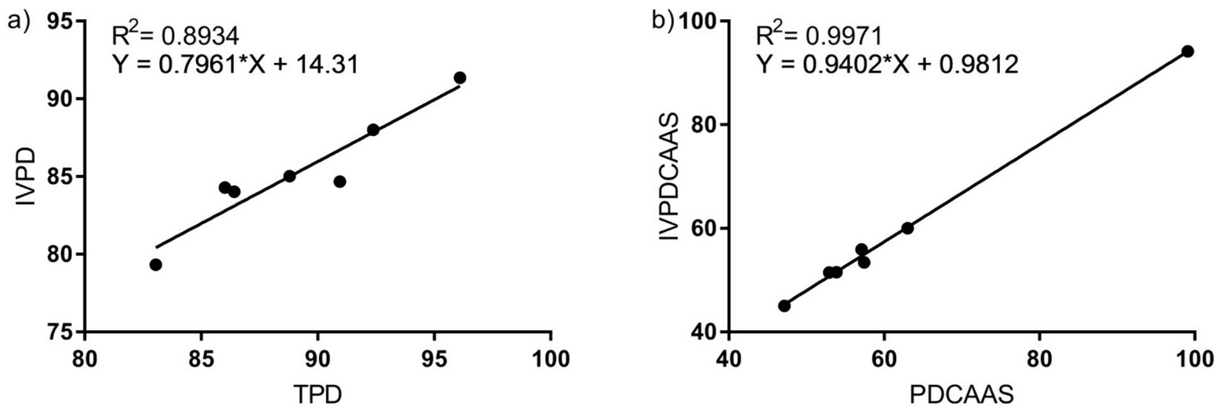

Fig. 1. Relationship between the digestibility, extruded, cooked and baked red and green lentil flours determined by *in vitro* and *in vivo* methods (a) and the relationship between the protein digestibility-corrected amino acid scores calculated using *in vitro* and *in vivo* digestibilities (b). TPD = true protein digestibility, IVPD = *in vitro* protein digestibility, PDCAAS = protein digestibility corrected amino acid score, IVPDCAAS = *in vitro* protein digestibility corrected amino acid score.

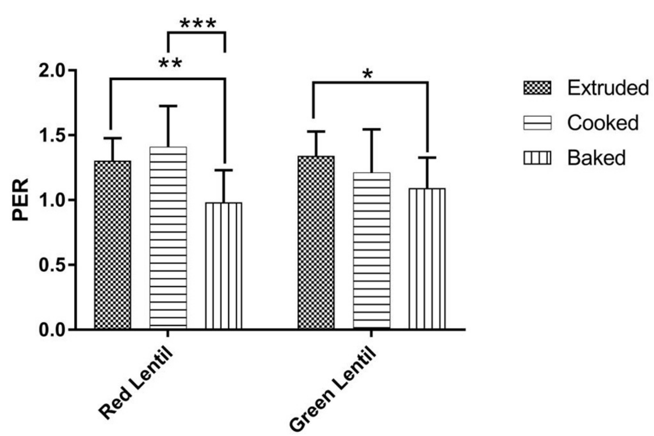

Fig. 2. Protein Efficiency Ratio (PER) values of extruded, cooked and baked red and green lentil flour. Hatched bars indicate baked flour, horizontal bars are cooked flour and vertical bars are extruded flour. Mean ± SD (n = 10). Data were analyzed via Two-Way ANOVA with Tukey's post hoc test. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3.4. Digestible Indispensable Amino Acid Score

In 2013 the FAO/WHO presented a new method for determining protein quality, the digestible indispensable amino acid score (DIAAS) (FAO/WHO, 2013). This method was designed to be a replacement for PDCAAS as it utilized ileal digestibility rather than fecal digestibility, individual amino acids were considered nutrients rather than protein and a different amino acid requirement pattern was constructed. As there is little data currently available on the ileal digestibility of amino acids, the FAO/WHO advised that fecal digestibility could be used in the calculation of DIAAS, which was done in this study. The DIAAS values for processed lentil flour ranged from 0.44 for baked green lentil to 0.58 for extruded red lentil. The pattern of DIAAS values were similar to those found in PDCAAS and *In Vitro* PDCAAS with extrusion having the highest value (0.58 for red lentil, 0.53 for green), followed by cooking (0.53 for red, 0.49 for green) and baking (0.50 for red, 0.44 for green). Although there is little DIAAS data currently available, one study found a value of 0.50 for cooked red lentils and 0.58 for cooked green lentils (Nosworthy, Franczyk et al., 2017). In all cases DIAAS values were lower than either PDCAAS or *In Vitro* PDCAAS. As the same amino acid profile and protein digestibility were used in the calculation of DIAAS and PDCAAS, this difference occurs due to the change in the amino acid reference pattern, particularly the sulfur amino acids. In calculating PDCAAS the requirement for sulfur amino acids is 25 mg/g protein while the DIAAS requirement is 27 mg/g protein (FAO/WHO, 1991, 2013). This increase in requirement reduced the amino acid value for DIAAS thereby causing the lower value compared to PDCAAS. Although it is the current recommendation that PDCAAS be replaced with DIAAS it is important to consider the impact of this shift on regulatory decisions. A recent review discussed the potential impacts of a shift to DIAAS on regulatory frameworks and came to the conclusion that using the suggested model, ileal-cannulated pigs, is not practical and the focus should rather be on using fixed estimates of ileal digestibility (similar to those currently in place for folate) or using *in vitro* analyses for determination of protein digestibility rather than *in vivo* (Marinangeli & House, 2017). A comparison between PDCAAS, *in vitro* PDCAAS and DIAAS illustrates the relative relationship between these measurements of protein quality. In all cases PDCAAS has the highest value, DIAAS the lowest and *in vitro* PDCAAS being intermediate. This indicates that, for this sample set, *in vitro* analysis of protein quality provides an average value between both PDCAAS and DIAAS.

3.5. Protein Efficiency Ratio

The protein efficiency ratio (PER) is the measurement of protein quality that is mandated by Health Canada in order to regulate protein content claims (Health Canada, 1981). This measurement is different