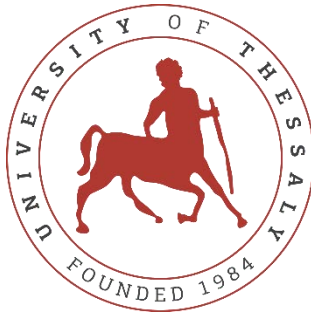




University of Thessaly

Faculty of Agricultural Sciences

Department of Animal Science

Improvement of Nitrogen Use Efficiency of Feed in Dairy Replacement Heifers in Greece

Antigoni Plomaritou

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University of Thessaly, Department of Animal Science

Antigoni Plomaritou

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IMPROVEMENT OF NITROGEN USE EFFICIENCY OF FEED IN
DAIRY REPLACEMENT HEIFERS IN GREECE

A dissertation presented by

Antigoni Plomaritou

Supervised by

Dr. Andreas Foskolos

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Department of Animal Science of

University of Thessaly

In fulfillment of the requirements for the degree of

Doctor of Philosophy

Three-member committee

Dr. Andreas Foskolos, Associate Professor, University of Thessaly

Dr. Dimitrios Kantas, Professor, University of Thessaly

Dr. Eleni Dovolou, Associate Professor, University of Thessaly

Seven-member committee

Dr. Eleni Dovolou, Associate Professor, University of Thessaly

Dr. Andreas Foskolos, Associate Professor, University of Thessaly

Dr. Dimitrios Kantas, Professor, University of Thessaly

Dr. Panagiotis Plageras, Professor, University of Thessaly

Dr. Basdagianni Zoe, Assistant Professor, Aristotle University of
Thessaloniki

Dr. Eleni Tsiplakou, Professor, Agricultural University of Athens

Dr. Sokratis Stergiadis, Professor, University of Reading

*Στους γονείς μου,
Κωνσταντίνο και Γεσθημανή,
για όλες τις θυσίες που έχουν κάνει.*

Ευχαριστίες

Το κομμάτι αυτό της διατριβής, θα μπορούσε να είναι και το μεγαλύτερο, αφού δεν θα μπορούσα να είχα φτάσει στο τέλος χωρίς την υποστήριξη και βοήθεια πολλών ανθρώπων. Δεν ξέρω αν η ευγνωμοσύνη μου μπορεί να εκφραστεί με τις διαθέσιμες λέξεις, ωστόσο θα ήθελα πρωταρχικά να ευχαριστήσω τον επιβλέποντα καθηγητή μου, Δρ. Ανδρέα Φώσκολο. Η καθοδήγησή, υποστήριξη, προσοχή, υπομονή, επιμονή, παρατηρήσεις και ενθάρρυνσή του ήταν καθοριστικοί παράγοντες για την διεκπεραίωση αυτής της διατριβής. Έδειξε εμπιστοσύνη στις ικανότητές μου και αυτό είναι κάτι που δεν ξεχνώ. Επίσης, είμαι βαθιά ευγνώμων για την αμέριστη συμπαράσταση και κατανόησή του σε σημαντικές στιγμές της ζωής μου, δυσάρεστες αλλά και χαρούμενες.

Προχωρώντας, θα ήθελα να ευχαριστήσω όλους εκείνους που με την επιστημονική τους κατάρτιση, τις συμβουλές και τη βοήθεια τους, διαμόρφωσαν την διατριβή αυτή. Θα ήθελα να ευχαριστήσω, λοιπόν, την Δρ. Ζωή Μπασδαγιάννη για την βοήθεια της τόσο στην αρχή αυτού του ταξιδιού, αλλά και στην τελευταία πειραματική μου μελέτη. Επίσης, θα ήθελα να ευχαριστήσω τον Δρ. Δημήτριο Καντά και την Δρ. Ελένη Ντόβολου για τα σχόλια, τις παρατηρήσεις και την βοήθειά τους καθ' όλη την διάρκεια αυτής της διατριβής. Τέλος, θα ήθελα να ευχαριστήσω τον Δρ. Χρήστο Μακρίδη, τον Δρ. Θεμιστοκλή Γιαννούλη και την Δρ. Ναταλία Βασιλείου για τις πολύτιμες συμβουλές τους και την βοήθεια τους όποτε τις χρειαζόμουν.

Δεν θα μπορούσα ποτέ να παραλείψω όλους τους φοιτητές του Τμήματος μας, που όλα αυτά τα χρόνια μας βοήθησαν ώστε να ολοκληρωθούν

αμέτρητες ώρες αναλύσεων και αρκετές εκατοντάδες ημέρες δειγματοληψιών. Δειγματοληψίες που έγιναν συνήθως σε συνθήκες δύσκολες, με πολύ κρύο ή πολλή ζέστη, πολύ αργά το βράδυ ή πολύ νωρίς το πρωί, παλεύοντας με φτυάρια, ζυγαριές και, χωρίς ίχνος υπερβολής, μοσχίδες. Για αυτούς τους λόγους και με την ελπίδα να μην ξεχάσω κανένα θα ήθελα να ευχαριστήσω τους: Κωνσταντίνο Γατσά, Στέλιο Αθανασιάδη, Κωνσταντίνο Τρέμμα, Θεοδώρα Μίχου, Κωνσταντίνο Δρουμτσέκα, Αλέξανδρο Τσίγκα, Λαυρεντία Καρατοσίδη, Γιάννη Βακόνδιο, Αγγελική Χαριοπολίτου, Νικόλα Κούση, Ηλέκτρα Γλάρου, Παρασκευή Γκίκα, Αναστασία Τζαμουράνη, Άννα Καρακώστα, Κωνσταντίνο Φραγκάκη, Σόνια Τσαγκλή, Χρήστο Πλιάκα, Βασίλη Βασιλείου, Αναστάσιο Κακαρούγκα, Βασίλη Γαλάνη. Δεν θα ήθελα να παραλείψω, επίσης και όλους τους κτηνοτρόφους, οι οποίοι με χαρά μας υποδέχτηκαν στις εκτροφές τους συμβάλλοντας και αυτοί στην προσπάθειά μας. Ειδικότερα, ένα ευχαριστώ είναι λίγο για την οικογένεια Φραγκάκη, η οποία σύσσωμη μας φιλοξένησε κατά την διάρκεια της τελευταίας πειραματικής μελέτης. Οι αναμνήσεις αυτές, όπως και το γεμιστό κοτόπουλο θα μείνουν για πάντα.

Λίγο πριν το τέλος, θα ήθελα να ευχαριστήσω την οικογένεια μου. Χωρίς την στήριξη, ηθική και οικονομική, την αγάπη και πίστη τους σε εμένα, αυτό το ταξίδι δεν θα είχε ολοκληρωθεί. Σας ευχαριστώ, λοιπόν, από καρδιάς· ακόμη και αν δεν είναι όλοι παρόντες, είμαι σίγουρη πως είστε περήφανοι για εμένα! Επίσης, ευχαριστώ τον σύζυγό μου Γιάννη, για την υποστήριξή, την υπομονή και ανοχή του στα δύσκολα ωράρια, αλλά και για την προσπάθειά του να καλύψει την απόσταση, να στέκεται δίπλα μου πάντα βράχος. Τέλος, οφείλω ένα μεγάλο ευχαριστώ στους φίλους μου και τη Ράιλι, τετράποδο συνοδοιπόρο από το προπτυχιακό ως το διδακτορικό.

List of abbreviations

Chapter 1:

AA, amino acids; ADG, average daily gain; AFC, age at first calving; AFOLU, agriculture, forestry and other land use; ATP, adenosine triphosphate; BCS, body condition score; BW, body weight; CNCPS, Cornell Net Protein and Carbohydrate System; CO₂, carbon dioxide; CO, carbon monoxide; CH₄, methane; CI, calving interval; CP, crude protein; DM, dry matter; DMI, dry matter intake; EBW, empty body weight; eqCO₂, CO₂ equivalent; EU, European Union; F:C, forage to concentrate ratio; GHG, greenhouse gas; GWP, global warming potential; HGC, heart girth circumference; LNE, lifetime nitrogen use efficiency; LULUCF, land use, land-use change and forestry; MBW, mature body weight; ME, metabolizable energy; MNE, milk nitrogen use efficiency; MP, metabolizable protein; NDF, neutral detergent fiber; N, nitrogen; NFC, non-fiber carbohydrates; N_r, reactive nitrogen; N₂, di-nitrogen; NH₃, ammonia; NH₄⁺, ammonium; NUE, nitrogen use efficiency; NO₂, nitrogen dioxide; NO_x, nitrogen oxides; NO₃⁻, nitrates; N₂O, nitrous oxide; NPN, non-protein nitrogen; O₃, tropospheric ozone; PM, particulate matter; R², coefficient of determination; RDP, rumen degradable protein; RUP, rumen undegradable protein; SP, soluble protein; SO₂, sulfur dioxide; TP, true protein; VOC, volatile organic compounds.

Chapter 2-5:

ADG_{1stSer}, ADG from weaning to first service; ADG_{SerCal}, ADG from first service to first calving; ADG_{1stCal}, ADG from weaning to calving; T_{1stSer}, age at first service; T_{1stCal}, age at first calving; BHBA, beta-hydroxybutyrate acid; Bact_{SC}, structural carbohydrates fermenting bacteria; Bact_{NSC}, non structural

carbohydrates fermenting bacteria; BW_{1stSer}, BW at first service; BW_{1stCal}, BW at first calving; EE, ether extract; HCP, high crude protein treatment; HNM, holistic nutritional management; HRDP, high rumen degradable treatment; LCP, low crude protein treatment; LRDP, low rumen degradable treatment; N_{Bal}, nitrogen balance; N_{Fc}, nitrogen contained in feces; N_{Int}, nitrogen intake; N_{Urn}, nitrogen contained in urine; NH₃-N, ammonia nitrogen; NEFA, non esterified fatty acids; OM, organic matter; TMR, total mixed ration; uNDF, undigestible neutral detergent fiber.

Περίληψη

Διάφορες πρακτικές στον τομέα της γεωργίας, όπως η εκτεταμένη χρήση λιπασμάτων και η εναπόθεση κοπριάς στις γεωργικές εκτάσεις, για την αύξηση της παραγωγικότητας των καλλιεργειών, διατάραξαν τον κύκλο του αζώτου (N). Αυτό οδήγησε στο φαινόμενο, γνωστό ως καταρράκτης N, το οποίο περιγράφει τον σχηματισμό και την κινητοποίηση ανόργανων δραστικών μορφών N (N_r) στα διάφορα οικοσυστήματα. Οι μετακινήσεις και ανταλλαγές N_r μεταξύ των εδαφικών, ατμοσφαιρικών και υδάτινων οικοσυστημάτων προκάλεσαν πολλές αρνητικές περιβαλλοντικές επιπτώσεις (όξυνση εδάφους και υδάτων, ευτροφισμό, συνεισφορά στην δημιουργία αερίων του θερμοκηπίου) και αναφέρονται ως νιτρορύπανση. Η ζωική παραγωγή και, ιδιαίτερα, η γαλακτοπαραγωγός βοοτροφία θεωρείται ένας από τους τομείς που συμβάλλουν σημαντικά στην νιτρορύπανση.

Η αύξηση αποδοτικότητας χρήσης N (AXN) των αγελαδοτροφικών εκτροφών, έχει προταθεί ως λύση κλειδί για την άμβλυνση του προβλήματος της νιτρορύπανσης. Οι μοσχίδες αντικατάστασης αποτελούν σημαντικότερο κομμάτι των γαλακτοπαραγωγικών κοπαδιών, με την συμβολή τους στην νιτρορύπανση να μην μπορεί να θεωρηθεί αμελητέα. Η ηλικία του πρώτου τοκετού και οι απώλειες N κατά την διάρκεια της περιόδου ανάπτυξης επηρεάζουν αρνητικά την συνολική AXN των εκτροφών. Επομένως, ο στόχος της παρούσας διδακτορικής διατριβής ήταν να περιγράψει τις πρακτικές εκτροφής των μοσχίδων αντικατάστασης που επηρεάζουν την AXN της τροφής των μοσχίδων αντικατάστασης και να διερευνήσει διατροφικούς παράγοντες που μπορεί να βελτιώσουν την AXN της τροφής, μειώνοντας τις απώλειες N στο περιβάλλον. Προς επίτευξη του στόχου αυτού, διεξήχθησαν τρεις μελέτες.

Στην πρώτη μελέτη, διερευνήθηκαν οι συνθήκες διαχείρισης και η τρέχουσα κατάσταση της AXN της τροφής των μοσχίδων αντικατάστασης γαλακτοπαραγωγικής κατεύθυνσης στην Ελλάδα. Συνολικά συλλέχθηκαν δεδομένα από 14 εκτροφές γαλακτοπαραγωγικής κατεύθυνσης. Κατά την επίσκεψη διάρκειας 2 ημερών σε κάθε εκτροφή, συλλέχθηκαν πληροφορίες σχετικά με την διαχείριση όλης της εκτροφής, αλλά και αυτή των μοσχίδων αντικατάστασης. Έτσι, δημιουργήθηκαν 3 βάσεις δεδομένων για την αξιολόγηση της αναπαραγωγής, της ανάπτυξης και των σιτηρεσίων. Για κάθε εκτροφή, ανακτήθηκαν αναπαραγωγικά στοιχεία όλων των αγελάδων της εκτροφής και η ηλικία της πρώτης αναπαραγωγής (T_{1stSer}), τεχνητές σπερματεγχύσεις (ΤΣ)/μοσχίδα και η ηλικία πρώτου τοκετού (T_{1stCal}) προσδιορίστηκαν. Στα διάφορα αναπτυξιακά στάδια, το σωματικό βάρος (ΣΒ) για το 15-20 % όλων των ομάδων των μοσχίδων εκτιμήθηκε με χρήση μετρικής ταινίας και υπολογίστηκε η αντίστοιχη μέση ημερήσια αύξηση βάρους (MHAB). Επιπλέον, για την αξιολόγηση της ανάπτυξης, εκτιμήθηκε το ενήλικο σωματικό βάρος (ΕΣΒ) των αγελάδων κάθε εκτροφής. Στόχοι ανάπτυξης τέθηκαν με βάση το ΕΣΒ κάθε εκτροφής ως εξής: (1) 55% ΕΣΒ στην πρώτη αναπαραγωγή, (2) 85% ΕΣΒ στον τοκετό ($Target_{85}$) και (3) 95% ΕΣΒ στον τοκετό ($Target_{95}$). Επιπρόσθετα, τέθηκαν οι αντίστοιχοι στόχοι MHAB για κάθε αναπτυξιακό στάδιο. Για την αξιολόγηση των σιτηρεσίων, καταγράφηκαν τα σιτηρέσια ολικής ανάμειξης που χορηγούνταν σε όλες τις ομάδες των μοσχίδων και συλλέχθηκαν δείγματα των επιμέρους ζωοτροφών, τα οποία και αναλύθηκαν χημικά. Η αξιολόγηση των σιτηρεσίων έγινε με την χρήση του διατροφικού μοντέλου Cornell Net Carbohydrate and Protein System (CNCPS v6.5). Βρέθηκε ότι η T_{1stSer} και T_{1stCal} ήταν 15,4 ($\pm 2,4$) και 25,3 μήνες ($\pm 2,65$), αντίστοιχα. Οι

μοσχίδες χρειάστηκαν 1,54 ΤΣ/μοσχίδα για να συλλάβουν. Το ΣΒ των μοσχίδων στην T_{1stSer} και T_{1stCal} ήταν 437,1 κιλά and 692,8 κιλά, αντίστοιχα. Τα ΣΒ ήταν εντός των στόχων του 55 % και 85 % ΕΣΒ (404 και 625 κιλά για T_{1stSer} και T_{1stCal}, αντίστοιχα), ωστόσο η έναρξη της αναπαραγωγής έγινε με ένα μήνα καθυστέρηση. Σε όλες τις περιπτώσεις, η μεταβολιστέα ενέργεια ήταν ο πρώτος περιοριστικός παράγοντας. Η μεταβολιστέα πρωτεΐνη (ΜΠ) χορηγήθηκε σε πλεόνασμα υπερβαίνοντας τις ανάγκες των μοσχίδων, με το πλεόνασμα ΜΠ να αυξάνεται με την αύξηση της ηλικίας των μοσχίδων ($P < 0.01$). Για την περίοδο 3 έως 12 μηνών, η AXN ήταν υψηλότερη (μέσος όρος 26.1 %), ενώ από 15 έως 24 μηνών η AXN μειώθηκε (μέσος όρος 13,0 %). Συμπερασματικά, συχνή παρακολούθηση του ΣΒ και προσαρμογή της διατροφικής διαχείρισης ανάλογα με το αναπτυξιακό στάδιο και τις ανάγκες των μοσχίδων είναι αναγκαία προκειμένου να αποφευχθεί η χορήγηση περίσσειας ΜΠ.

Στην δεύτερη μελέτη, διερευνήθηκε η επίδραση του επιπέδου ολικών αζωτούχων ουσιών (ΟΑΟ) και πεπτής πρωτεΐνης στη μεγάλη κοιλία (ΠΠ) στην AXN της τροφής των μοσχίδων αντικατάστασης. Η μείωση του επιπέδου των ΟΑΟ χωρίς να επηρεαστεί η παραγωγικότητα (ανάπτυξη, γαλακτοπαραγωγή) ή η μεταβολή της αναλογίας μεταξύ ΠΠ και μη πεπτής πρωτεΐνης στη μεγάλη κοιλία (ΜΠΠ), έχουν προταθεί ως διατροφικές στρατηγικές για την μείωση της απέκκρισης του Ν στο περιβάλλον και βελτίωση της AXN της τροφής. Η μελέτη περιέλαβε 2 δοκιμές πεπτικότητας σε πειραματικό σχεδιασμό διασταύρωσης, με δύο επεμβάσεις σε 8 μοσχίδες ($n = 8$) η κάθε μία. Στις μοσχίδες ανατέθηκε τυχαία μία από τις 2 επεμβάσεις. Στην πρώτη δοκιμή, 2 επίπεδα ΟΑΟ (14,9 % ΞΟ; HCP και 12,1 % ΞΟ; LCP) χορηγήθηκαν στις μοσχίδες. Στην δεύτερη δοκιμή, τα σιτηρέσια περιείχαν δύο διαφορετικά επίπεδα ΠΠ (75,2 % ΟΑΟ;

HRDP και 64,3 % ΟΑΟ; LRDP) με ένα χαμηλό επίπεδο ΟΑΟ. Οι μοσχίδες στεγάστηκαν ατομικά. Κατά την διάρκεια της δειγματοληπτικής περιόδου, καταγράφηκε η χορηγούμενη προσφερόμενη ποσότητα και ποσότητα της άρνησης του σιτηρεσίου και λήφθηκαν δείγματα της προσφοράς, άρνησης, ζωοτροφών, κοπράνων, ούρων, υγρού μεγάλης κοιλίας και αίματος. Στην πρώτη δοκιμή, η κατανάλωση N ήταν υψηλότερη για την επέμβαση HCP σε σύγκριση με την LCP (164,9 έναντι 130,4 γρ/ημέρα, αντίστοιχα, $P < 0.01$). Η περιεκτικότητα των ούρων σε N ήταν υψηλότερη για τις μοσχίδες HCP (60,8 και 43,1 γρ/ημέρα για HCP και LCP, αντίστοιχα, $P < 0.01$). Η υψηλότερη κατανάλωση N είχε την αντίστοιχη επίδραση στο αμμωνιακό N του υγρού μεγάλης κοιλίας (12,6 και 8,1 mg/dL για HCP και LCP, αντίστοιχα, $P = 0.03$) και στα επίπεδα ουρίας του αίματος (4,8 και 3,3 mmol/L για HCP και LCP, αντίστοιχα, $P < 0.01$). Το ισοζύγιο N ήταν υψηλότερο για τις μοσχίδες της HCP από ότι στις μοσχίδες της LCP (48,3 και 34,8 γρ/ημέρα για HCP και LCP αντίστοιχα, $P = 0.03$), ωστόσο δεν παρατηρήθηκε κάποια επίδραση του υψηλότερου επιπέδου ΟΑΟ στην AXN της τροφής. Στην δεύτερη δοκιμή, η κατανάλωση N δεν επηρεάστηκε από το διαφορετικό επίπεδο ΠΠ. Το αμμωνιακό N του υγρού μεγάλης κοιλίας ήταν υψηλότερο για την επέμβαση HRDP σε σύγκριση με την LRDP (10,9 και 4,5 mg/dL για HRDP και LRDP, αντίστοιχα, $P < 0.01$). Η ουρία στο αίμα ήταν υψηλότερη για τις μοσχίδες HRDP (5,4 και 3,7 mmol/L για HRDP και LRDP, αντίστοιχα, $P < 0.01$). Δεν παρατηρήθηκε διαφορά στην απέκκριση και το ισοζύγιο του N. Παρ' όλα αυτά, μία τάση για μειωμένη AXN της τροφής παρατηρήθηκε για τις μοσχίδες της HRDP (17,4 και 23,0 % για HRDP και LRDP, αντίστοιχα, $P = 0.10$). Συμπερασματικά, ακόμη και αν το υψηλότερο επίπεδο ΟΑΟ επηρέασε θετικά

το ισοζύγιο N, αυτό έγινε με υψηλότερη συγκέντρωση N στην μεγάλη κοιλία, οδηγώντας σε υψηλότερη απέκκριση του N στο περιβάλλον. Σε ένα σταθερό χαμηλό επίπεδο ΟΑΟ, το χαμηλότερο επίπεδο ΠΠ φαίνεται να μειώνει τις απώλειες N στο περιβάλλον μέσω των ούρων.

Στην τρίτη μελέτη, σκοπός ήταν η δημιουργία μίας ολιστικής προσέγγισης της διατροφής για την βελτίωση της AXN των γαλακτοπαραγωγικών εκτροφών, τόσο σε επίπεδο ζώου όσο και εκτροφής. Το σχήμα της ολιστικής διατροφικής διαχείρισης (HNM) απευθύνεται σε όλους τους ενδιαφερόμενους που εργάζονται την αύξηση της AXN. Η χορήγηση ΟΑΟ υπερβαίνοντας τις ανάγκες των ζώων, υπήρξε ένας τρόπος αντιμετώπισης της αβεβαιότητας σε ότι αφορά την διακύμανση της χημικής σύστασης των χονδροειδών ζωοτροφών, αλλά και των διατροφικών των αναγκών. Το πλεονασματικό N που χορηγείται στα ζώα, απεκκρίνεται στο περιβάλλον κυρίως μέσω των ούρων και σε λιγότερο βαθμό μέσω των κοπράνων. Γενικά, το επίπεδο των ΟΑΟ χρησιμοποιούνταν ως ένδειξη της χορήγησης N, ωστόσο η ΜΠ είναι ακριβέστερο μέτρο των πρωτεϊνικών αναγκών. Κύριες πηγές ΜΠ για το ζώο είναι οι μικροοργανισμοί της μεγάλης κοιλίας και η πρωτεΐνη της τροφής που προσπερνά την αποδόμηση στην μεγάλη κοιλία. Η μικροβιακή πρωτεΐνη είναι η φθηνότερη πηγή πρωτεΐνης για το ζώο, ωστόσο η μεγιστοποίηση της σύνθεσης της γίνεται όταν χορηγούνται επίσης επαρκή επίπεδα μεταβολιστέας ενέργειας. Όταν η σύνθεση της μικροβιακής πρωτεΐνης έχει μεγιστοποιηθεί, η παραγωγή αμμωνίας στην μεγάλη κοιλία μπορεί να μειωθεί, με αποτέλεσμα την μείωση της απέκκρισης N μέσω των ούρων. Επομένως, κατά την κατάρτιση σιτηρεσίων, προσπάθεια πρέπει να γίνεται για την μεγιστοποίηση της ΜΠ που

προέρχεται από τους μικροοργανισμούς, ενώ ενθαρρύνεται η ένταξη ζωοτροφών στα σιτηρέσια που είναι διαθέσιμες μετά την μεγάλη κοιλία.

Πέρα από τον μεταβολισμό του ζώου, διαφοροποιήσεις στις πρακτικές διαχείρισης μπορούν να βελτιώσουν περαιτέρω την AXN των εκτροφών. Η δημιουργία διατροφικών ομάδων με βάση την παραγωγικότητα εντός του κοπαδιού, είναι ένα βήμα προς την αύξηση της AXN στο γάλα και επομένως όλης της εκτροφής. Επιπλέον, η χρήση μηχανιστικών διατροφικών μοντέλων όπως το Cornell Net Protein and Carbohydrate System (CNCPS, v6.5) επιτρέπει την ακριβή πρόβλεψη των αναγκών και αποτρέπει πιθανές απώλειες των θρεπτικών στο περιβάλλον. Παράγοντες που αφορούν το ζώο, όπως γαλακτοπαραγωγή, σύνθεση γάλακτος, ΣΒ, δείκτης σωματικής διάπλασης και κατανάλωση ξηράς ουσίας είναι πολύτιμες εισροές σε τέτοιου είδους μοντέλα. Επομένως, η παρακολούθηση όλων των παραπάνω παραγόντων, η συχνή χημική ανάλυση των ζωοτροφών και η κατάλληλη εφαρμογή των σιτηρεσίων (απόδοση προσωπικού, ομοιογένεια σιτηρεσίου) συνιστάται να ενταχθούν στις πρακτικές διαχείρισης κάθε εκτροφής.

Summary

Several practices in agriculture, as the extended use of fertilizers and manure application on fields to increase cultivation productivity have disrupted nitrogen (N) cycle. This have led to the phenomenon N cascade, which describes the creation and mobilization of inorganic reactive N (N_r) forms in different ecosystems. The N_r transformation and moving through terrestrial, aquatic and atmosphere ecosystems have caused negative environmental effects (eutrophication, soil acidification, water acidification, GHG emissions) leading to N pollution.

Livestock production and, especially, dairy cattle contribute to N pollution. Increasing nitrogen use efficiency (NUE) of dairy cattle farms has been suggested as a key action towards N pollution mitigation. Dairy replacement heifers comprise a major part of dairy herds and their contribution to N pollution cannot be neglected. The age at first calving and N losses during the period of growth negatively impact overall farm NUE. Therefore, the aim of current PhD dissertation was to describe heifer rearing practices that affect NUE of replacement heifers and look into nutritional factors that may improve NUE and minimize N losses in the environment. To achieve the general aim of the current PhD thesis, three studies were conducted.

In the first study, we investigated the management conditions and the current status of NUE of dairy replacement heifers in Greece. We collected data from 14 dairy farms and obtained information about the overall farm and heifer rearing management during a 2-day visit in each farm. We built, three datasets: the reproduction, growth and ration evaluation datasets. For each farm, reproduction records of the herd were retrieved and the age of first service (T_{1stSer}), services/heifer and age of first calving (T_{1stCal}) were determined. We estimated body weight (BW) in different stages by the use of measuring tape at 15-20 % of all heifer groups and we calculated the respective stage's average daily gain (ADG). Moreover, in order to evaluate growth, the mature BW (MBW) of the cows in each herd was estimated. Growth targets based on farm's MBW were set as follows: (1) 55% MBW at first service, (2) 85% MBW at calving ($Target_{85}$) and (3) 95% MBW ($Target_{95}$) at calving. Moreover, the respective ADG targets for each growth stage were set. For ration evaluation, we recorded the total mixed ration (TMR) fed to all heifer groups and we collected feed

samples to be chemically analyzed. The ration evaluation was realized using the Cornell Net Carbohydrate and Protein System (CNCPS v6.5). We found that T_{1stSer} and T_{1stCal} was 15.4 (± 2.4) and 25.3 months (± 2.65), respectively. Heifers required 1.54 services/heifer in order to achieve pregnancy. Heifer BW at T_{1stSer} and T_{1stCal} was 437.1 kg and 692.8 kg, respectively. These BW were within the targets of 55 % and 85 % of MBW (404 and 625 kg for T_{1stSer} and T_{1stCal} , respectively), however, they were achieved with 1-month delay. The first limiting factor in all cases was metabolizable energy. Metabolizable protein (MP) % was supplied above heifers' requirements and increased along with heifers age ($P < 0.01$). From 3 to 12 months NUE was higher (on average 26.1 %), while from 15 to 24 months NUE decreased (on average 13.0 %). We concluded that frequent BW monitoring and adjustments in nutritional management should be implemented in each growth stage according to respective requirements to avoid MP oversupply.

In the second study, the effects of CP and RDP levels on N use efficiency (NUE) of dairy replacement heifers were examined. Lowering CP level without compromising growth and production or altering RDP: RUP ratio has been proposed as a nutritional strategy to decrease N excretion in the environment and improve NUE. The study comprised of 2 digestibility trials in a switchback design, with two treatments in 8 heifers ($n = 8$) each. Then, heifers were assigned randomly in one of two treatments. In first trial, 2 levels of CP (14.9 % DM; HCP and 12.1 % DM; LCP) were offered to heifers. In the second trial, formulated diets contained two different RDP levels (75.2 % CP; HRDP and 64.3 % CP; LRDP) in a LCP diet. Heifers were housed individually. During sampling period, we recorded offer and refusals weight and we collected offer,

refusal, feed, feces, urine, rumen fluid and blood samples. In the first trial, N intake was higher in HCP compared with LCP treatment (164.9 vs 130.4 g/d, respectively, $P < 0.01$). The urinary N was higher for HCP heifers (60.8 and 43.1 g/d for HCP and LCP, respectively, $P < 0.01$). The higher N intake had corresponding effects on rumen $\text{NH}_3\text{-N}$ (12.6 and 8.1 mg/dL for HCP and LCP, respectively, $P = 0.03$) and blood urea levels (4.8 and 3.3 mmol/L for HCP and LCP, respectively, $P < 0.01$). The N balance was higher for HCP than LCP heifers (48.3 and 34.8 g/d for HCP and LCP respectively, $P = 0.03$), however we observed no effect of the higher CP level on NUE. In the second trial, N intake was not affected, when the RDP level was altered. Rumen $\text{NH}_3\text{-N}$ was higher for HRDP compared with LRDP treatment (10.9 and 4.5 mg/dL for HRDP and LRDP, respectively, $P < 0.01$). Correspondingly, blood urea N was greater for HRDP heifers (5.4 and 3.7 mmol/L for HRDP and LRDP, respectively, $P < 0.01$). We observed no difference on N excretion and N balance. However, a tendency for decreased NUE was observed for heifers fed the HRDP treatment (17.4 and 23.0 % for HRDP and LRDP, respectively, $P = 0.10$). In conclusion, even though higher CP level affected positively N balance, higher N concentration in rumen was observed, leading to increased N excretion in the environment. A constant lower CP level and lower RDP protein level appear to reduce N losses in the environment via urine.

In the third study, we aimed to provide a holistic approach to improve NUE of dairy cattle farming at an animal and farm level. The holistic nutritional management (HNM) scheme is addressed to all stakeholders, who are working towards this goal. Feeding CP above requirements has been a strategy to deal with uncertainty regarding forage quality and animal's requirements. The

excessive N that is supplied to animals is excreted in the environment mainly in the form of urine and to a lesser extent contained in the feces. Traditionally, CP level of the diet has been used as an indicator of diet's N supply, however, MP is a more accurate measure in terms of protein requirements. Main sources of MP for the animal is the rumen microbes and rumen by-pass feed protein. Microbial protein is the cheapest source of protein; however, its synthesis can be maximized when adequate levels of ME are provided. When microbial protein synthesis is maximized, NH_3 production in the rumen can be reduced and as a consequence N excreted with urine is also reduced. Therefore, in ration formulation, effort should be made to maximize MP deriving from rumen microbes, while the incorporation of feed protein that escapes rumen degradation in the diets is encouraged.

Besides animal's metabolism, alterations in management practices can further improve farm NUE. The formation of different feeding groups within a herd based on groups' productivity is a step towards increase of milk NUE and, thus, farm NUE. Moreover, the use of mechanistic nutritional models, such as The Cornell Net Protein and Carbohydrate System (CNCPS, v6.5) allows the accurate requirement prediction and prevents potential nutrient loss in the environment. Animal parameters regarding milk production, milk composition, BW, BCS and DMI are valuable inputs in order to run such nutritional models. Therefore, monitoring all the above parameters in combination with frequent feed chemical analysis and proper ration implementation (feeder performance, ration's homogeneity) is suggested to be incorporated in farm's routine management practices.

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Chapter 1

Literature Review

1.1. Introduction

Increased public concern forces dairy sector to perform in a more sustainable way. Dairy cattle farming has been associated with nitrogen (N) pollution, as practices of the past involving agricultural and manure management, led in deposition of excessive N amounts into the ecosystem (Dijkstra et al., 2013; Fowler et al., 2013). Moreover, nutritional practices, such as overfeeding protein to maintain high levels of milk production, contributed to the problem (Firkins and Reynolds, 2005). The N quantity, which is not utilized by the animal, retained in the body or used for milk production, is excreted through urine and feces in the environment (Van Amburgh et al., 2015). The increase of N use efficiency (NUE) of dairy farming systems has been proposed as an action towards nitrogen pollution reduction (Sutton et al., 2011). The NUE is practically a fraction of N output divided by the N input into the system and at an animal level can be defined as g of N in product/g N consumed (Calsamiglia et al., 2010). Identifying all parameters that regulate NUE of the farms might further improve N utilization and minimize N losses in the environment (Calsamiglia et al., 2010).

Most efforts in mitigating N pollution target the lactating cows, with heifers often receiving less attention. Replacement heifers consist a major part of dairy herds, as they are kept as inventory for future cows (Boulton et al., 2017). Usually, heifers enter the milking herd at an age at first calving (AFC) of 2 years old. At a farm level, heifer's AFC appears one of the factors that highly affect lifetime nitrogen use efficiency (LNE) of the dairy farms, since higher AFC values have been identified as a factor that negatively impacts overall farm LNE

(Foskolos and Moorby, 2018). Thus, controlling AFC is crucial as a first step towards reduction of N losses.

The length of the rearing period is practically determined by the age that heifers are bred, while the beginning of the breeding process depends heavily on factors related to growth and nutrition (Le Cozler et al., 2008). Thus, the manipulation of the nutritional plan, through adjustments in the average daily gain (ADG), can be used to control age at first breeding and, thus, achieve a desired AFC. Towards this direction, the definition of specific growth targets based on each growth stage and growth monitoring may lead to reduced AFC. Moreover, heifer nutrition and especially the metabolizable protein to crude protein ratio has been correlated with NUE of replacement heifers (Foskolos and Moorby, 2018). Improving heifer's N consumption and N retention in heifer's body, thus improving overall N utilization, along with reduced rearing periods may further minimize N losses.

Therefore, proper reproduction and nutritional management of heifers enable the programming of first calving and might both serve as a tool to mitigate and improve overall farm NUE.

1.2. Importance of dairy replacement heifers

Whether to cull or not a dairy cow is an important and rather complex decision for dairy farmers to make. Several parameters should be taken into consideration, such as production systems, current cow performance, replacement availability, and quality of replacements to ensure future productivity (De Vries and Marcondes, 2020). The reasons for culling a dairy cow are classified as voluntary (e.g. low productivity, market prices, etc.) or

involuntary (e.g. reproductive problems, health issues, etc.; Fetrow et al., 2006). The number of cows being removed due to poor milk production has been decreased, over the past years, with the main reason for culling being infertility (Compton et al., 2017). Moreover, increased calf mortality (Brickell and Wathes, 2011) and the fact that one third of animals only calve once (Wathes et al., 2008), make the decision even more complex. It is considered that a cow exiting the herd would be replaced timely, however this decision is highly depended upon the availability, quality and economic benefit of the replacement heifers (Overton and Dhuyvetter, 2020).

The extend at which cows are culled is described by the culling rate, which determines the percentage of cows leaving the herd (Hadley et al., 2006). In Table 1.1 the culling rates of different countries are presented, ranging from 21,3% in Ireland to 40% in Sweden. From data presented in Table 1.1., an average culling rate value of 30.8 % can be calculated. This means that a 100-cow dairy herd needs approximately 31 replacement heifers to enter the milking herd annually. It is obvious that as culling rate increases, more heifers should be reared to maintain herd size. The rearing of replacement heifers consists the second largest cost of milk production (15-20% of total cost), following nutrition cost of lactating cows (Heinrichs, 1993; Tozer and Heinrichs, 2001). The total rearing cost of raising a heifer has been determined at £1.819, €1.567, \$1.808 in the UK in 2013, The Netherlands in 2010 and USA in 2011, respectively (Mohd nor et al., 2012; Heinrichs et al., 2013; Boulton et al., 2017). However, because of the recent societal and economic events, this cost is expected to be higher.

Table 1.1. Culling rate of dairy cows in different countries.

Country	Culling rate	Reference
Canada	30.4%	CDIC (2024)
Estonia	33.0%	Rilanto et al. (2022)
France	21.5%	Raboisson et al. (2011)
Germany	28.9%	Habel et al. (2021)
Ireland	21.3%	Maher et al. (2008)
Italy	34.0%	Bellingeri et al. (2019)
Netherlands	21.9%	Kulkarni et al. (2023)
Poland	30,8%	Pytlewski et al. (2010)
Scotland	33.7%	Chiumia et al. (2013)
Spain	26.0%	Bugueiro et al. (2019)
Sweden	35-40%	Alvåsen et al. (2018)
USA	36.0%	De Vries (2017)

The time required to offset rearing cost has been found to be approximately 1.5 lactation periods (Boulton et al., 2017). Figure 1.1, presents the time point at which enough revenue is generated in order to recover expenses that were created during the rearing process (Oikonomou, personal communication). It is obvious, that this breakeven point in the cow's lifetime appears approximately at the beginning of the third lactation, with the economic return being apparent afterwards. However, this offset might never actually happen, as not all heifers reach second lactation period. Indeed, Brickell and Wathes (2011) reported that from the total number of heifers that reached first calving, 19% was culled in the first lactation period, while only 55% of total

heifers reared, reached third calving. Therefore, increasing culling rates negatively impact overall farm profitability, as the associated heifer rearing cost increases as well.

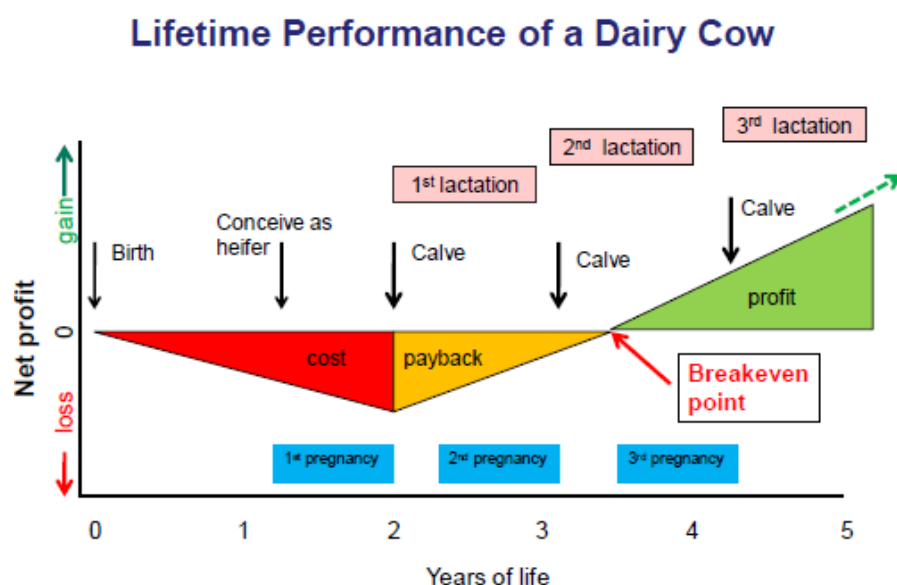


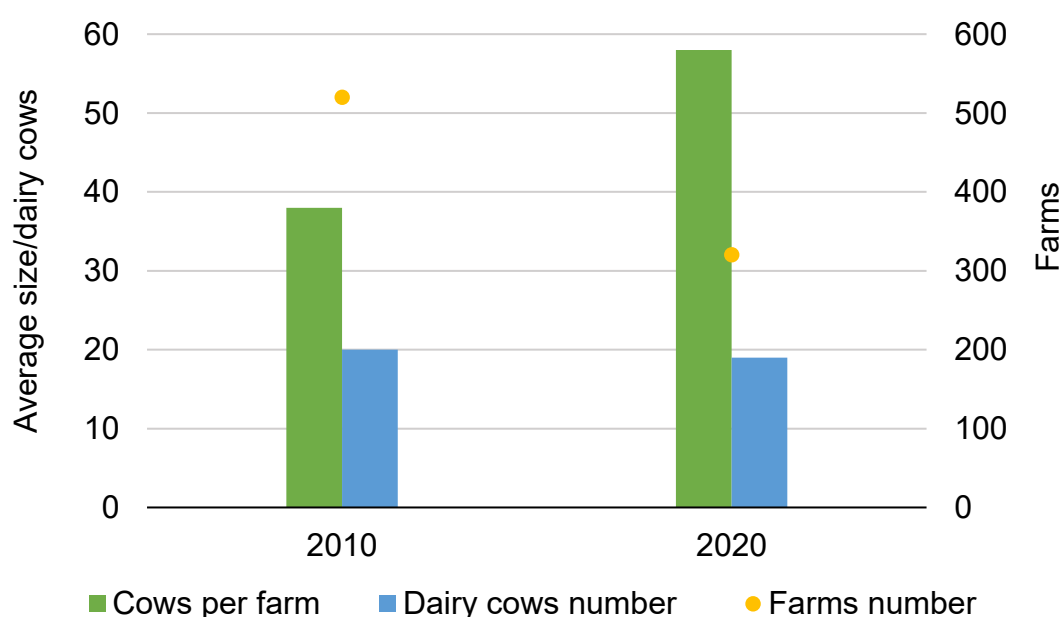
Figure 1.1. Cow lifetime profitability and revenues to payback the rearing associated costs (Oikonomou, personal communication).

Culling does not always represent high mortality rates or poor welfare (Overton and Dhuyvetter, 2020). For example, a high culling rate is reported for the USA (Table 1.1). Therefore, increased culling rates may reflect the effort to genetically improve the herd taking into consideration also economic factors (Overton and Dhuyvetter, 2020). Genomic testing of dams and sires allows the determination of superior animals of the farm and helps identify animals that need to be bred or culled (Overton and Dhuyvetter, 2020). As a next step, superior animals would be selected to create higher genetic merit replacements. In the majority of the cases worldwide, source of dairy

replacements is the farm itself (Gabler et al., 2000). In addition, common practices are considered to be the direct purchase of pregnant heifers (Tozer and Heinrichs, 2001) or contract raising, in which heifers of a farm are sold or transferred to another to get raised and then they are purchased or received back (Overton and Dhuyvetter, 2017). In any case, either heifers are farm-raised or purchased, heifer genomic testing has been used to accurately identify heifers that should be bred (Clasen et al., 2021). Indeed, genomic testing of heifers along with the use of sexed semen has found to positively impact herd's overall genetic gain (Bérodier et al., 2019). Moreover, analysis of growth performance has been suggested as tool to select replacement heifers that meet farm's productivity goals (Overton and Dhuyvetter, 2020). Even though heifers represent a significant cost of milk production, it is clear that they consist an investment for the future success of the dairy business rather than an absolute expense. Therefore, a culling plan thoughtfully implemented both to cows and heifers, serves to genetically improve the herd.

Over the last 60 years herd size and milk yield per cow have been increased (Dallago et al., 2021) through advances in nutrition, health, management and genetic selection (Shook, 2006). In the European Union (EU), the abolition of milk quotas in 2015 changed the structure of dairy farms, as an extreme increase of herd size was observed in an effort to increase milk production and profit from growing milk product demand (Gonzalez-Mejia et al., 2018; Kelly et al., 2020). For example, in the UK between 2010 and 2020, average herd size increased from 121 to 155 cows per farm (AHDB, 2024), while in Ireland an increase of 23 % was observed in herds maintaining more than 100 cows in 2016 (Kelly et al., 2020). This trend was also observed in

almost all developed countries (Barkema et al., 2015). However, as it shown in Figure 1.2, over the years the number of dairy farms decreased dramatically, dairy cows number decreased slightly, while there is a noticeable increase of cows per farm (EC, 2022). This trend is well known as consolidation and intensification of dairy farms. In this case many dairy farms were closed down, while the rest of the farms increased their herd size and milk production (Gonzalez-Mejia et al., 2018). Besides the opportunity for higher milk production, increased herd size has been found to positively impact technical



economic efficiency, improving the profitability of farms (Krpalkova et al., 2016; Siafakas et al., 2019).

Figure 1.2. Average dairy cows' (million) number, farm (thousand) number and farm size (cows/farm) in European Union in 2010 and 2020 (EC, 2022).

In conclusion, other than replacing what is culled, replacement heifers serve as an inventory that enable the genetic improvement and size increase of the herd. Proper heifer rearing management can be considered as a strategy for dairy farms to remain economically sustainable and an opportunity to increase overall farm profitability.

1.3. Growth management of dairy replacement heifers

Main goal of replacement heifer management is raising healthy and good quality heifers with the lowest possible cost (van Niekerk et al., 2021). From a physiological point of view, dairy replacement heifers do not enter the milking herd until calving. This period lasts approximately 2 years or even more, with average AFC being reported at 24.5, 26.1 and 29.1 months, in USA, Italy and UK, respectively (Hutchison et al., 2017; Eastham et al., 2018; Ferrari et al., 2024). It is obvious, that an increased AFC, extends non-productive period, increases rearing cost and delays further the pay-back time of raising process (Tozer and Heinrichs, 2001; Boulton, 2015b).

Indeed, AFC has been shown to impact farm profitability, with AFC at 24 months maximizing profits (Ettema and Santos, 2004; Mohd Nor et al., 2013). Moreover, at 24 months heifers are considered grown and mature enough to calve with no difficulties and achieve a good performance in first lactation period (Boulton, 2015a). Hutchison et al. (2017) observed that AFC at 24 months resulted in higher levels of milk production in first lactation period, but lifetime milk production and conception rates were higher at the AFC of 21 to 22 months. Eastham et al. (2018) showed that highest lifetime milk production occurred at an AFC of 22 months (15.25 kg/d, ranging 15.16-15.35 kg/d). Moreover, even though a further reduction of AFC at 21 months resulted in the

lowest milk yield, milk protein and fat content in first lactation period, this was not the case in terms of lifetime daily milk yield, with heifers that calved at 21 producing more milk through their lifetime than heifers that calved at 24 months. Moreover, this kind of approach is desirable and beneficial in systems that are maintaining a seasonal pattern of milk production. Seasonal calving systems are usually pasture based, hence breeding and calving are restrained in a short period of time, where pasture availability meets nutrient requirements (Grosshans et al., 1997; Handcock et al., 2021). Thus, heifers at this type of systems calve at a specific age, usually 24, 30 or 36 months (Le Cozler et al., 2008).

Overall, even though currently the rearing goal is AFC at 24 months, in near future AFC may further decrease at the lowest possible age without compromising health and productivity. Rearing programs should focus on reducing the non-productive period, while ensuring that heifers will obtain the appropriate frame size without compromising milk yield in first lactation period (Van Amburgh et al., 1998b). Thus, management practices oriented towards controlling AFC are useful and may lead to improved farm profitability.

1.3.1. Management circle of heifers' growth

Rearing process is a long lasting one with many morphological and physiological changes. The “two-year circle of excellence” describes the sequence of these changes realized during heifer rearing period of life (Figure 1.3; Jones and Kammel, 2017). Based on the different nutritional, housing and management requirements the following stages can be distinguished:

(1) *Stage 1*: birth to weaning, this period lasts approximately 2 months, but is highly depended on the weaning program,

(2) *Stage 2*: weaning to 4 months old, period in which calves are becoming functional ruminants. This period is considered as the transition period, that is from liquid to solid feed, and lasts until calves reach 100-125 kg of body weight (BW; NRC, 2001; NASEM, 2021).

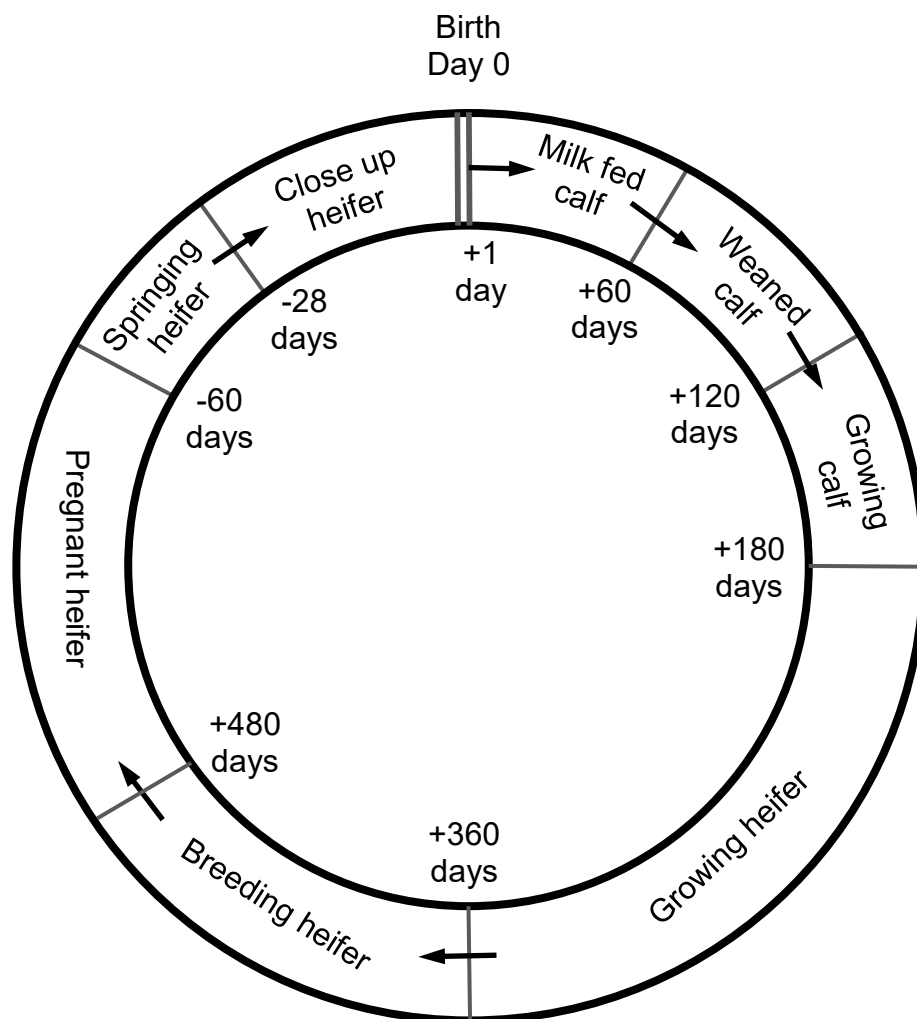


Figure 1.3. Two-year circle of replacement heifer rearing (Jones and Kammel, 2017).

(3) *Stage 3*: 5 months to 12 months old, which is characterized by the onset of puberty and by the end heifers are considered reproductively mature. However,

onset of puberty is a breed related trait (Le Cozler et al., 2008). In big sized dairy breeds, puberty usually appears around 9-11 months (Boulton, 2015b).

(4) Stage 4: 13 months to 15 months old. At this stage, heifers enter the reproduction process. It is evident, that to achieve the target of AFC at 24 months at a herd level, breeding should start earlier than 15 months to include any reproduction failures, such as returns or embryo losses in early gestation (Le Cozler et al., 2008).

(5) Stage 5: 16 months to 22 months old. During this period heifers are pregnant, with gestation lasting on average 9 months or 279.4 ± 5.7 days (Norman et al., 2009).

(6) Stage 6: 23 months to 24 months. These are the two last months of pregnancy with which the circle of excellence is completed. Heifer becomes a primiparous cow with the borne calf entering the circle.

Specific growth benchmarks should be met to achieve AFC at 24 months of age. The growth targets for replacement heifers are expressed as a percentage of the mature body weight (MBW). It should be noted that MBW is estimated as the mean BW of herd's third lactation cows, as dairy cows reach mature size at third calving (Le Cozler et al., 2008; Hoffman, 2017). Body size is a heritable characteristic (Coffey et al., 2006), while within a farm the animal selection might be towards large or small frame size (Hoffman, 2017). Thus, growth targets are strongly related to herd and breed (Handcock et al., 2019a).

The required BW in different growth benchmarks is determined by coefficients based on MBW. Van Amburgh et al. (1998b) as a BW target for first calving has proposed at least the 82 % of MBW. Fox et al. (1999) according to

a prior model (Fox et al., 1992) and modifying existed data from Van Amburgh et al. (1998b), suggested BW at 85 % MBW after calving, and 92 and 96 % MBW as target weights for second and third parity, respectively. The National Research Council (NRC; 2001) based on the recommendations of Fox et al. (1999), proposed BW of 82, 92 and 100 % MBW for first, second and third calving, respectively. Hoffman (2017) based on adapted data from Hoffman (1997) and NRC (2001), suggested as breeding and prepartum BW target the 55 % and 94 % MBW. Van Amburgh et al. (2019) concluded that enhancing first lactation period milk yield is feasible when heifer BW postpartum ranges between 82 and 85 % MBW. Recently, National Academies of Science, Engineering and Medicine (NASEM) suggested 55 % MBW as breeding BW goal and 82% MBW as the target BW for first calving following previous edition of NRC (Table 1.2; (NRC, 2001; NASEM, 2021).

Table 1.2. Coefficients of growth targets expressed as percentage of mature body weight (MBW) for dairy replacement heifers based on different suggestions.

Stage	MBW%				
	Van Amburgh et al., 1998b	Fox et al.,1999	NRC, 2001	Hoffman, 2017	NASEM, 2021
Breeding	55-60	-	55	55	55
Prepartum	-	-	-	94	91
1 st Calving	82	85	82	-	82
2 nd Calving	-	92	92	-	92
3 rd Calving	-	96	100	-	100

At first calving, in an effort to minimize nutrient utilization towards growth, increased BW is considered essential (Van Amburgh et al., 2019). Additionally, lighter heifers often exhibit lower conception and pregnancy rates (Ferrell, 1982; Wathes et al., 2008), while it is observed that heavier heifers (291-316 kg) at breeding calve earlier compared with heifers of lower BW at breeding (≤ 290 kg; Archbold et al., 2012). Handcock et al. (2020) highlighted the importance of increased BW attainment before breeding and demonstrated that increased BW at 6, 12 and 15 months old increased heifer survival up to third lactation and likely induced earlier calving compared to lighter heifers. However, a slight decrease was observed in reproductive performance and survival of heifers belonging at upper BW range compared to mid-range heifers (Handcock et al., 2020).

Moreover, increased BW at first calving is suggested, as a positive relationship between first lactation milk production and BW at first calving has been reported (Van Amburgh et al., 1998b). Handcock et al. (2019b) examined the effects of BW at calving to milk production from first to third lactation period. They found a positive curvilinear relationship between milk yield and BW and reported that for a heifer calving at 21 months old an increase in BW at first calving from 425 to 450 kg, resulted in an increase of 168 and 509 kg of energy corrected milk, in first and third lactation period (Handcock et al., 2019b). Han et al. (2021) examined the effect of heifer BW at calving to milk yield at first lactation and at 24 months after first calving analyzing records from 1,110 and 1,229 cows of two different herds (Penn State University herd; PSU and University of Florida herd; UF, respectively). The milk yield at first lactation period and 24 months after first calving was analyzed by a linear model taking

into consideration the effects of BW quintiles at calving, MBW and BW changes occurring in the first month of milk production. The BW at calving ranged from 477-624 kg and 473-604 kg, while the BW as a percentage of MBW ranged 68.9-87.9 % and 67.8-85.9 % for PSU and UF herd, respectively. Heaviest heifers yielded higher in milk production during the first lactation period (10,034 kg and 9,683 kg for 5th and 1st quintile, respectively), while no difference was observed in milk yield 24 months after first calving. Additionally, heaviest heifers lost more BW during first month of milk production (3.65 and 3.99 % BW change for heifers of 4th and 5th quintile, respectively) and had higher risk of culling due to significant BW loss. Thus, in contrast with other research findings, Han et al. (2021) suggested heifers to calve at 73-77% MBW (1st and 2nd quintile heifers) and concluded that any increase in BW after the 3rd quintile of BW was not associated with first lactation milk yield. More recently, Lauber and Fricke (2023) demonstrated that primiparous cows that had attained 92 and 85% of MBW post-calving had higher mean milk production at 8 weeks of lactation compared to cows attaining the 75 and 81 % of MBW post-calving (38.4 and 36.7 kg/d, and 33.8 and 35.9 kg/d, respectively). However, similar to the study of Handcock et al. (2020), heifers who have attained the 92 % of MBW post-calving calved 20 days later than leaner heifers (75 % MBW) and failed to conceive at first breeding (Lauber and Fricke, 2023).

Overall, the determination of cows' MBW and the establishment of growth targets at benchmarks is important for allowing heifers to express their genetic potential. There are indications suggesting that by reaching MBW already from the first lactation period, other parameters can be compromised in the cost of higher milk production, such as survival and conception rates.

Moderate-high BW post-calving at 85% of MBW appears to benefit first milk production levels without negative implications.

1.3.2. Measures of growth

Growth from birth until calving can provide insight into heifer rearing efficiency (Bazeley et al., 2016). The quantification of growth through BW measurement and ADG estimation, is the simplest indication of developmental maturity (White et al., 2015). The most accurate way to measure BW is a weighing scale (Dingwell et al., 2006); however, its absence in commercial farms is a common phenomenon (Wood et al., 2015). Alternatively, equations that are including body measurements (e.g. heart girth circumference (HGC), hip width, body length and withers height) are used to estimate BW (Heinrichs et al., 1992). In Holstein breed heifers, HGC is highly correlated with BW ($R^2 = 0.9873$) and by using a metric tape around animal's heart girth BW can be estimated by the following equation (Heinrichs et al., 1992; Heinrichs et al., 2007):

$$BW = 65.36 - (1.966 * HGC) + (0.01959 * HGC^2) + (0.00001691 * HGC^3),$$

where BW is measured in kg and HGC in cm.

This equation accurately predicts the BW of heifers and especially those who weigh more than 150 kg, while the estimation differs from actual weight by less than 8% (Heinrichs et al., 2007). For example, in the case of a heifer that actually weighs 300 kg, the expected BW range obtained by the equation is from 288 to 312 kg. However, it has been found that for heifers younger than 3 months old the use of measuring tape results in lower BW estimation than the actual BW (Dingwell et al., 2006).

Another measure for the evaluation and control of growth, is the absolute growth rate or ADG. The ADG is the BW difference over a specific period of time and can be calculated as follows:

$$ADG = \frac{BW_2 - BW_1}{t_2 - t_1},$$

where ADG is measured in kg/day, BW_1 is initial BW in kg at t_1 and BW_2 is latest BW in kg at t_2 .

Heifer's BW and ADG are both parameters that can be easily estimated on farm and become part of heifer management routine. Growth monitoring is essential to evaluate heifer rearing efficiency and enables decision making to ensure that heifers can achieve their genetic potential.

1.3.3. Growth curves

Simple growth indicators, as ADG and BW, offer the opportunity to adjust management practices. However, they do not provide information about the trend that growth follows over time. Thus, growth models have been developed to describe the lifetime relationship between BW and age, in an effort to interpret the biological meaning of growth (Fitzhugh, 1976).

Table 1.3. Functions of nonlinear models describing growth curves.

Function	Body weight ¹
Gompertz	$Ae^{(-be^{-kt})}$
Von Bertalanffy	$A(1-be^{-kt})^3$
Brody	$A(1-be^{-kt})$
Logistic	$A(1+be^{-kt})^{-1}$
Richards	$A(1-be^{-kt})^M$

¹ A = asymptotic value of MBW curve in kg, b = integration constant, k = maturing index, t = time in days, M = proportion of final size at inflection point.

Nonlinear models such as Gompertz, Von Bertalanffy, Brody, logistic and Richards model have been used to model changes in growth (Table 1.3; (Fitzhugh, 1976; Perotto et al., 1992). Richards model is a function combining the rest four models. In Figure 1.4, examples of non-linear functions to model heifer growth are depicted. This kind of models integrate MBW (A; asymptote of the curve) and growth rate (k; maturing index that describes the rate at which BW reaches MBW, M; inflection point of sigmoid curve that establishes the transition between accelerating and inhibiting phase of growth; (Fitzhugh, 1976)).

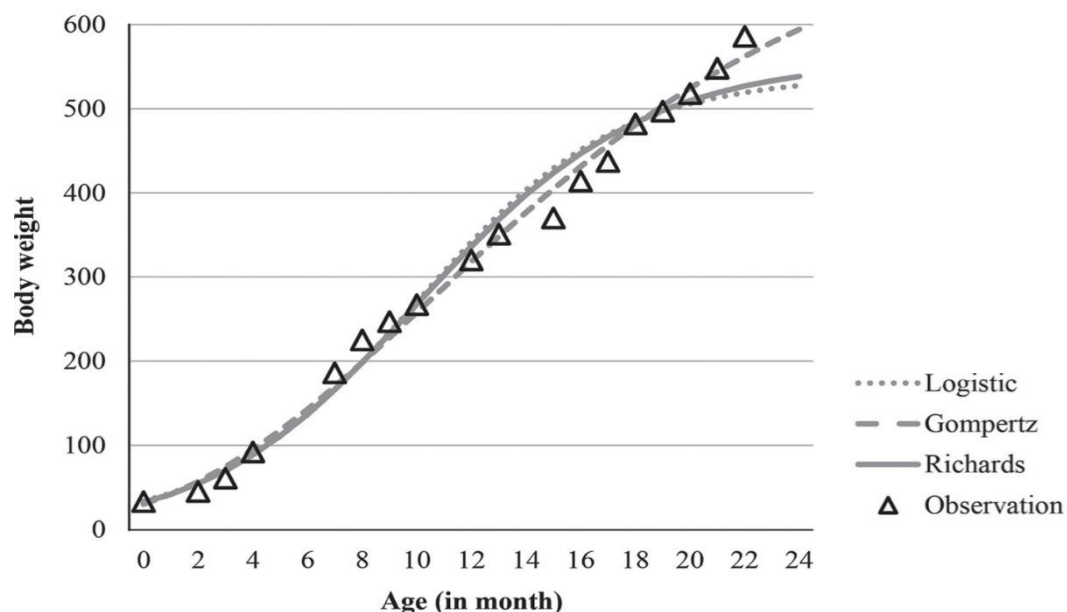


Figure 1.4. Non-linear growth functions to model the growth curves of dairy heifers (Yin and König, 2020).

Perotto et al. (1992) compared growth functions (logistic, Gompertz, Brody and Richards) and concluded that growth curves of female dairy cattle

are better described by the Richards model. Nonlinear models regress size on age. However, it should be noted that age is not the only parameter that promotes growth and pushes an organism to mature (Fitzhugh, 1976). Other factors such as nutrient consumption, feed availability and changes in productivity level can create variation in the relationship of BW with age (Fitzhugh, 1976). Moreover, nonlinear models assume that growth until the inflection point is linear and require the asymptote of MBW to project the growth curve (Handcock et al., 2019a).

When repeated measurements through the lifetime of the animal are possible, MBW can be determined. However, when heifer population is examined, usually, measurements are not recorded until first calving. Therefore, linear models have been considered more suitable than nonlinear models to create growth curves of growing heifers (Cue et al., 2012). Cue et al. (2012) in Canada obtained a large dataset of national BW and height recordings. Heifers were still in the ascending phase of growth and MBW asymptote was not known. Hence, Cue et al. (2012) created a linear random regression model to project growth curve. Similarly, Silva et al. (2021) used mixed effect linear regression to describe the relationship between BW and age. In both studies of Cue et al. (2012) and Silva et al. (2021) it was observed that BW data of younger animals were less dispersed than older animals' BW, as weight gains in the earlier life were more consistent. Even though BW data of younger ages had lower standard deviation than older ones, goodness of fit (R^2) for Silva et al. (2021) was 0.859, while for Cue et al. (2012) power of mean estimate and residual variance was reported at 1.2 and 0.26, respectively. Moreover, Handcock et al. (2019a) in pasture-based systems, when seasonal

fluctuations required to be considered, showed that random regression using Legendre polynomials predicted BW accurately with relative prediction error of 4%. Thus, nonlinear or linear models are used according to each case and the data availability (e.g. known MBW).

Overall, the use of growth curves allows the prediction of future heifer growth. Prediction of BW at benchmarks, such as breeding, can limit high-risk decisions and provide confidence that heifers have attained the proper BW to be bred at a specific age or otherwise delay breeding process due to insufficient BW. Therefore, growth curves along with BW monitoring can serve as a tool to improve and modify management practices.

1.4. Growth and nutritional management of dairy replacement heifers

1.4.1. Physiological basis and impacts of nutrition on heifer growth and productivity

Growth is not a steady state, thus, understanding the factors that are involved during this process is really important. Different genetic, biological and physiological aspects, such as developmental plasticity, body composition and nutritional requirements are involved through this process.

It is generally accepted that a phenotypic characteristic, such as milk yield, is determined by genotype and environment. For example, proper nutrition would provide the substrate for animal's genetic potential to be expressed. However, the concept of developmental plasticity supports that a certain genotype can express different phenotypes without altering DNA sequence under epigenetic modifications, starting already at the time of conception (Hochberg et al., 2011; Funston and Summers, 2013). Moreover, in

the last decades, research has shown that the level of cow performance is influenced even earlier than birth. Several dam related factors such as nutrition, number of parities, exposure to heat stress appear to affect heifer performance, starting from prenatal stage of life and continuing until puberty (Garcia et al., 2016; Monteiro et al., 2016). Based on this mechanism, prenatal and postnatal life, nutritional and management strategies directed to dam and calf, respectively, may impact subsequent heifer growth and development.

From a management point of view, heifer rearing programs are subdivided in several stages as it is shown in Figure 1.3. However, growth and development are two interdependent processes that continue to progress until maturity. It is obvious that each growth stage relies on the previous one, with early life establishing the foundation of a successful rearing process. Early life programming is considered to define heifer growth response in the subsequent stages. For instance, increased colostrum intake right after birth positively affected growth rate until first calving, while milk production increased until second lactation period (Faber et al., 2005). The high levels of ADG from birth to weaning appear to be a main driver of growth in high levels also after weaning (Soberon et al., 2012), and be responsible for the attainment of puberty at young age (Abeni et al., 2019). Moreover, preweaning ADG appears to impact the level of first lactation milk production (Soberon et al., 2012; Bazeley et al., 2016). Indeed, it has been found that preweaning ADG explained the 22% of milk yield variation observed in the first lactation period of two different herds, since for each kg of ADG, milk production was increased by 850 kg and 1113 kg (Soberon et al., 2012).

Nevertheless, the effects of nutrition on growth should be evaluated taking into consideration body composition. Body composition, as well as composition of gain is defined mainly from feeding level and degree of maturity (NRC, 2001). Lean tissue deposition from preweaning period until around 200 kg of BW, is pretty constant and higher than fat deposition (Figure 1.5; Van Amburgh et al., 2019). However, protein accretion declines after 30 % MBW (NASEM, 2021).

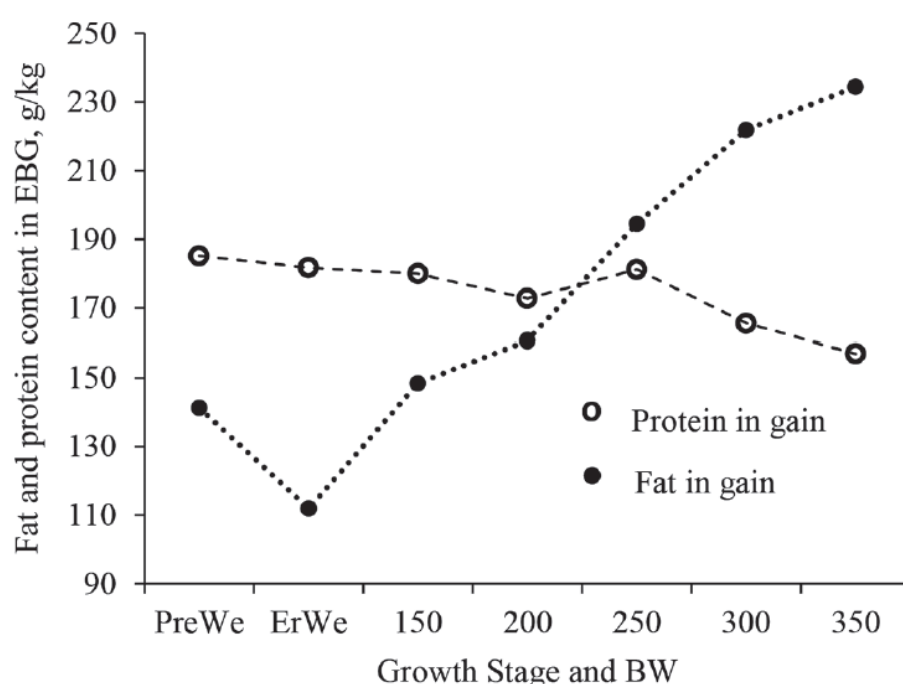


Figure 1.5. Fat and protein composition of empty body gain of replacement heifers in different life stages (Van Amburgh et al., 2019).

The period before puberty is considered the one with the highest feed efficiency after weaning and transition period (Bach and Ahedo, 2008), while the largest part of heifer growth being completed until conception (Boulton et al., 2015). As it shown in Figure 1.5, after 250 kg BW, protein gain of empty BW (EBW) slightly decreases while fat content of EBW increases significantly (Van

Amburgh et al., 2019). Hence, the retention of protein related to fat decreases as the heifers reach maturity, meaning that weight gain is comprised mainly by fat, while the protein content of gain is minimized (NASEM, 2021). In other words, as BW increases along with an increasing intake, body crude protein (CP) retention is limited by body fat and if energy exceeds maintenance requirements, the excessive energy will be deposited as fat, diluting other body components. However, the chemical body composition highly depends on mature size, meaning that composition of same BW animals might differ (NASEM, 2021). Body composition and composition of gains allow the nutritional program to target the specific requirements and benefit from efficient nutrient utilization of each growing phase, without nutrient losses.

In conclusion, heifer rearing programs should allow the expression of heifer genetic potential. Towards this direction, early life programming and proper nutritional management at each growing stage are essential.

1.4.2. From birth to weaning

Typically, the weaning of young calf is a process where calf is separated from the dam, transitioned from liquid to solid feed and adjusted gradually to social interactions (Weary et al., 2008). Calves that are allowed to suckle their dams, consume 6-12 kg of milk per day with high daily meal frequency (Khan et al., 2011b). Without external intervention, calves of bovidae family are weaned at the age of 6, 9 and 12 months, for beef cattle, Zebu and water buffalo calves, respectively (Reinhardt et al., 1978; Tulloch, 1979; Rasby, 2007). In dairy cattle, natural weaning of calves occurs between 6 to 9 months of age (Weary et al., 2008). As calves grow and become more independent, maternal care and milk flow are reduced and calves start to ruminate (Flower and Weary,

2003; Khan et al., 2011b). At 3 weeks old calves begin to graze and ruminate, while around the age of 4 to 6 months graze together with the older cows of the herd (Key and Maciver, 1985).

However, this kind of extended weaning is not implemented in modern farming systems. Within the first 24 hours after birth, the immediate calf's separation from the dam is realized (Stěhulová et al., 2008), while calves are abruptly or, less often, gradually weaned (Fischer et al., 2019). A common time frame for calves to be weaned is around 8 to 9 weeks of age or even earlier, with early weaning taking place as soon as 4 to 6 weeks (Fischer et al., 2019). Weaning is usually limited in a brief period of time to decrease nutritional cost of this phase (Fischer et al., 2019). In conventional weaning programs the amount of milk provided is restricted to a level of 10 % of calf birth weight, meaning 4 L of milk for a calf weighing 40 kg (Eckert et al., 2015). Moreover, according to NASEM (2021), calves should be introduced to solid feed as early as the first week of life. Therefore, conventional weaning programs aim to achieve early weaning, by reducing milk intake and encouraging solid feed intake so better rumen development could be achieved (Tamate et al., 1962). Based on this practice due to reduced milk intake, feeding cost is lower (Fischer et al., 2019). However, the BW gain is decreased due to milk restriction with a level of ADG at 0.3-0.5 kg/d (Jasper and Weary, 2002), while welfare concerns are raised (Khan et al., 2011b). Eckert et al. (2015) conducted a study including 20 Holstein heifers and observed lower stress levels when weaning took place at 8 weeks versus 6 weeks, while growth rate, nutrient intake and digestive tract development were better. Moreover, during the week of reduced milk intake, which for calves weaned at 6 weeks began at 36 days and for the ones weaned

at 8 weeks began at 50 days, ADG was lower for the calves of 6 weeks compared to 8 weeks program (0.35 kg/d vs 0.79 kg/d, respectively). This difference became bigger during the last week of weaning, where the calves weaned at 8 weeks (83.5 kg) had 3 times higher ADG, compared to the ones weaned at 6 weeks (66.5 kg), while this difference was maintained until 70, 90 and 150 days of age (Eckert et al., 2015). Therefore, the weaning method and the amount of milk consumed by the calves have great impact on welfare, performance, behavior and health (Khan et al., 2007). Nevertheless, it is noted that although ADG is nutritionally controlled, is also affected by events of disease, environmental factors (temperature and humidity), number of dam lactation period and litter size (Shivley et al., 2018).

Over the last years, there has been noted a transition from a nutritional plan of low or intermediate amount of milk intake to a plan of higher intake (elevated nutritional plan), which is closer to the expected amount of milk the dam would naturally provide to the calf (Mirzaei et al., 2018). An elevated nutrition plan is providing double the amount of milk or milk replacer compared to a conventional plan, which is considered 20% of the birth weight or approximately 8 L for a calf with birth BW of 40 kg (MacPherson et al., 2016). Usually at this feeding approach, the BW gain is considerably higher than conventional programs. Soberon et al. (2012) showed that weaning at 49 days (7 weeks) is feasible by elevating the nutritional plan above maintenance requirements, achieving that way double birth weight, from 41 kg to 82 kg, with an ADG of 0.82 kg/day. In the beginning of their life, calves depend totally on milk intake to meet maintenance and growth demands (Khan et al., 2016), therefore nutrient composition and quality of milk consumed is extremely

important to achieve desired BW gains (Moore et al., 2009). A milk replacer used in elevated nutritional programs contains 28% crude protein (CP) and 14-20% fat (Soberon et al., 2012), whereas a conventional milk replacer could contain 21,5% CP and 21,5% fat (Davis Rincker et al., 2011). Overall, the high levels of recommended CP, both in conventional and elevated weaning programs are based on the efficiency of lean tissue deposition and therefore on increased protein body deposition (Van Amburgh et al., 2019).

Weaning occurs when starter consumption is adequate, thus calves are introduced to starter as early as the first week of their life continuing the milk consumption (NASEM, 2021). In contrast with nature, where milk and pasture provide the stimulants for rumen development, in intensive farming systems development of rumen highly depends on solid feed provided (Khan et al., 2016). Solid feed initiates the development of rumen, through development of rumen papillae, which mainly occurs after the presence of fermentation products and mainly butyrate, that is considered the main epithelial stimulant (Sander et al., 1959). It is reported that the provision of around 5% chopped forages along with starter encourages consumption and utilization, especially when starter is provided as a pellet (Kertz et al., 2017). Moreover, heifers offered starter along with hay before weaning, developed better rumens compared to heifers offered only starter, while intakes were improved after weaning (Khan et al., 2011a; Khan et al., 2012).

The starter must be relatively rich in crude protein at 16-22% of dry matter (DM; Overton and Dhuyvetter, 2017). According to NASEM (2021), for a 80 kg calf fed milk replacer and starter at a ratio of 40:60, the daily requirements to sustain an ADG of 0.8 kg/d are 6.47 Mcal/d of energy and a

CP level at 20 % of DM. As starters are mainly grain-based (Khan et al., 2016), they contain high amounts of easily fermentable carbohydrates to stimulate rumen papillae development, while contain molasses to increase palatability (Kosiorowska et al., 2011; Overton and Dhuyvetter, 2017). However, in calves fed a starter containing ground grain and high level of starch, have been noticed undesired effects such as the alteration of gene expression that regulate rumen pH and rumen papillae shape (Beharka et al., 1998; Laarman et al., 2012). During transition period, solid feeds offered do not supply the same amount of protein that milk or replacers provide (Molano et al., 2020). Therefore, a reduction of ADG is normal during the last week of weaning, because of the increase in starter intake and the reduction of milk consumed (Davis Rincker et al., 2011).

After weaning heifers are transitioning from a diet with high concentrate levels to a forage based one. For a short period of about 1-2 months, starter is offered in limited amounts, while forage consumption is encouraged, until heifers are fully transitioned to a total mixed ration (Overton and Dhuyvetter, 2017). However, it is noted that in order not to suppress dry matter intake (DMI), forage neutral detergent fiber (NDF) should be provided at 0.89-1% of BW (Hoffman et al., 2008; Mitchell and Heinrichs, 2020), as high amounts of forage may decrease ADG and increase age at puberty (van Niekerk et al., 2021).

In conclusion, weaning programs should take into consideration weaning criteria such as starter intake or weaning age in order to reduce stress levels. Moreover, starter composition and solid feed provision should ensure proper rumen development. An elevated nutritional plan with high ADG, appears to

define the growth levels of subsequent growth stages, while affect the time of puberty's attainment and positively impact first lactation milk yield.

1.4.3. From weaning to age at first service

A crucial period that affects greatly the whole farm profitability is this of weaning until first service. At this period balancing rations for energy is of highest importance, as adipose tissue accumulation in the mammary gland due to excessive energy supply may lead to reduced milk yield at first lactation period (Van Amburgh et al., 1998b; Van Amburgh et al., 2019). Moreover, during this period heifers reach puberty and the time of conception determines the herd's AFC. Hence, the rearing efficiency of this period substantially can improve or negatively impact the economic stability of the farm.

Farmers usually decide to start the breeding process of heifers, based on age, as BW monitoring might be avoided either because of practical difficulties or unwillingness (Bach et al., 2021). However, when to start breeding process it not a matter of one factor, but it is rather an integration of parameters. A primiparous cow rather young in age or with low BW will partition nutrients towards growth instead of milk production at first lactation period (Van Amburgh et al., 2019). In contrast, greater AFC, as a result of older ages at breeding, impacts heifer survivability and lifetime milk production (Eastham et al., 2018). In addition, an overconditioned heifer with extended degree of fatness will probably exhibit metabolic diseases same as mature cows with high body condition score (BCS) at calving (Van Amburgh et al., 2019), while the ideal BCS for heifers at breeding is 3.0 in a 5-point scale (NASEM, 2021). It is, therefore, a necessity that heifers combine proper age and body size (BW and height) to start breeding (Wathes et al., 2014).

Even when heifers reach the desired age for breeding, they might not have the adequate BW and height due to slower growth rates. The ADG is highly affected by nutritional plan implemented, with the energy density of the diets resulting in higher or lower ADG (Cooke et al., 2013). Therefore, growth rates can be adjusted to achieve the desired BW goal. For example, in Figure 1.6, the effect of different growth rates on BW is shown. Heifers at 18 months presented difference in body size, as they were reared with growth rates set at 0.4, 0.6 and 0.8 kg/d (from left to right). Towards this direction, the nutritional management can ensure the attainment of adequate body size in growth benchmarks, such as age of first breeding.



Figure 1.6. Heifers of 18 months old grown at different growth rates set at 0.4, 0.6 and 0.8 kg/day (Van Amburgh et al., 2019).

Growth rates after weaning appear to affect age at puberty, AFC and milk production (Schillo et al., 1992; Funston et al., 2012; Heinrichs et al., 2017). Abeni et al. (2019) investigated the potential of late born heifers (between

December to February) to reach same BW targets with heifers born earlier (between September-November). They divided heifers into 3 groups, with 2 groups of heifers born between September-November being fed a standard (SD) and intensive diet (ID1), while the late born group (ID2) was fed the same intensive diet as ID1 group. Until 6 months of age all heifer groups were fed the same diet at different allowances and by the time heifers were introduced to pasture a supplementary diet was offered to allow ADG of 0.8 kg/d to SD and ID1 group and 0.9 kg/d to ID2 group. It was observed that BW was not affected by diets until 6 months, however after at 9 months the ID2 group showed higher BW (301 kg vs 265 and 262 kg for SD and ID1, respectively) with BW difference being maintained until 15 months, while attained puberty 1 month earlier (9.2 months vs 10.5 and 10.7 months for SD and ID1, respectively). Therefore, the feeding intensity chosen not only affects growth performance, but also the onset of puberty (Abeni et al., 2019). Several studies have examined the prepubertal ADG effect on mammary gland development and milk yield. Krpáľková et al. (2014) examined the effect of ADG, BW and BCS on milk yield until third lactation period. It was found that heifers that gained more than 0.95 kg/d from 5 to 14 months, produced more milk through the three lactation periods, while heavier heifers calved one month earlier and had a BCS of 3.5 at calving. A metanalysis of Zanton and Heinrichs (2005) demonstrated that an ADG of 0.8 kg/d in the prepubertal period, maximized milk production and milk protein content in first lactation, whereas ADG above 0.9 kg/d found to negatively impact first lactation milk yield. At high energy intake with gains of 1-1.1 kg/d or above, energy is not deposited as protein but rather as adipose tissue (Van Amburgh et al., 2019). However, it should be noted that mammary gland

development and thus, milk yield performance might not be compromised only by the ADG (Bach et al., 2021). Caution should be given in the composition and ratio of nutrients provided, meaning the balance between energy and protein should be taken into consideration to control fat and lean tissue deposition (Bach et al., 2021). Overall, when high growth rates at 0.8-0.9 kg/d are implemented through pubertal period, a reduction at age of breeding is feasible, leading to lower AFC without compromising milk production.

As mentioned earlier, in current stage control of diet energy supply is of high importance. In an effort to regulate intake and avoid overconditioning, provision of high amounts of low quality forage (Erickson et al., 2020) has been proposed as a method to dilute the energy content of the diet, especially when a corn silage based diet is offered to heifers (Coblentz et al., 2015), or limit-feed heifers at 70-90% of ad libitum DMI (Heinrichs et al., 2017; Hoffman, 2017). However, NDF intake appears to determine the level of DMI, with increasing NDF content limiting the DMI. Therefore, diets offered to heifers at the age of breeding is suggested to contain a level of NDF at around 1% of BW or at 44.5 % DM of the ration provided (Hoffman et al., 2008).

Moreover, non-adequate levels of metabolizable energy (ME) may negatively impact growth, regardless of CP level (Gabler and Heinrichs, 2003; Su et al., 2017). NASEM (2021) developed a new equation that takes into consideration heifer's BW and MBW and defined the minimum level of the MP supply (g/Mcal ME) resulting in 47.2 g MP/Mcal and 39.5 g MP/Mcal for a 150 and 350 kg heifer, respectively (NASEM, 2021). It is noted that N requirements on a MP basis for heifers growing with 0.84 kg/d and 700 kg MBW and weighing 224 and 420 kg are 610 and 772 g/d MP, respectively (NASEM, 2021).

As a conclusion, a high ADG during this period affects the onset of puberty and secures that heifers have attained proper BW at the first service. Consequently, a reduced age at first breeding shortens rearing period and thus reduces AFC. Even though it might be considered challenging, high growth rates should ensure that no excessive fat is deposited, as it may compromise future milk production.

1.4.4. From age at first service to age at first calving

After conception, heifer rearing program is mainly focused on maintaining adequate growth levels to support the heifer and fetus developmental requirements. According to NRC (2001), until 190 days after conception there are not any fetal requirements. This was based on the fact that most of the fetal growth is realized from mid to late gestation period, while studies used to develop protein and energy requirements were conducted from 190-270 days after conception (NRC, 2001). A recent slaughter study suggested that pregnancy requirements need to be accounted at 70 days in gestation (Sguizzato et al., 2020), while according to NASEM (2021) at 12 days in gestation, even though at this point requirements are reported as low as 1.6 g/d of metabolizable protein (MP).

Based on NRC (2001), a cow prepartum has MP requirements for maintenance of 480 g/d and 340 g/d for gravid uterus development. However, Bell et al. (2000) demonstrated that in fact MP requirements during late stages of pregnancy are higher, as MP for development of mammary gland was not taken into consideration, resulting in additional requirements of MP at about 120 g/d. According to NASEM (2021), a heifer 40 days prepartum at 80% MBW (700 kg MBW) has requirements of MP around 1672 g/d, 486 g/d for

maintenance, 250 g/d for pregnancy and 936 g/d to maintain an ADG of 0.7 kg/d, while the recommendations for dietary CP is at 13.5 % DM. In addition, Husnain and Santos (2019) conducted a meta-analysis and demonstrated that increasing prepartum CP levels of the diet from 12 to 15% and MP from 500 g/d to 1100 g/d, resulted in increased milk yield, milk fat and protein and DMI pre and post calving of primiparous cows, in contrast with multiparous cows in which an increase in dietary CP content did not bring further improvement of milk yield and DMI. As a consequence, based on NASEM (2021) and Husnain and Santos (2019), heifers calving for first time require additional protein for lean tissue deposition, resulting in higher protein requirements than multiparous cows.

Heifers after puberty have lower energy demands compared to heifers younger than 12 months and require about 2.1 Mcal/kg of DM (NRC, 2001). In an effort to control feed and energy intake, a method that has been proposed is limit feeding, leading to better utilization of nutrients and a reduction of feeding cost (Hoffman, 2017). Hoffman et al. (2007) fed 3 groups of Holstein heifers a control diet (CTR) with a CP level of 11.3 % DM and ME at 2.46 Mcal/kg of DM and two groups were limit fed at 90 and 80 % of DMI of control group, with CP and energy level set at 12.7 % DM and 2.55 Mcal of ME and 14.2% DM and 2.69 Mcal of ME, respectively. They found that for heifers fed the 80 and 90 % of CTR diet, no difference was observed on N allocation, heifer's BW and BCS, even though N retention was lower only arithmetically (35.2, 39.5 and 42.4, at 80, 90 and 100 % of CTR diet DMI, respectively). They concluded that limit feeding may be as efficient as energy diluted diets, as DM and NDF intake was lower, while feed efficiency was better, offering the

opportunity for decreasing nutritional cost of this phase. Similarly, Kruse et al. (2010) used heifers from 15 months old until 90 days in milk to detect any consequences of limit feeding on first lactation period. Diets offered to heifers at 100 % of DMI (control diet) contained CP at 13.1 % DM and 2.1 Mcal/kg of ME, at 85 % of the control diet contained CP level of 15.5 % DM and 2.4 Mcal/kg of ME and at 80% of the control diet contained the same composition as the 85% diet and were supplemented with lasalocid sodium. Through growing phase both limit-fed heifer groups exhibited better feed efficiency and improved ADG, as limit fed diet was an intense energy one. Limit-fed heifers exhibited tendency for increased BW with no difference on the frame size, increased BCS compared to control diet fed ones (3.2 versus 2.9, for limit fed and control group heifers, respectively), suggesting that limit fed heifers may deposited more adipose tissue. Through lactation period they reported that no differences were found on calf's BW, milk composition, production level, BCS and BW (Kruse et al., 2010). However, in Hoffman et al. (2007) study, limit fed heifers spend more time standing even without eating, vocalized more until adapting to the program, while Kruse et al. (2010) did not investigate the behavioral changes caused by the restricted provision of diet. Besides the welfare issues that arise from this management practice compared to ad libitum feeding, limit feeding is based on the concept of provision high nutrient diets. In the experiments mentioned above, even though diets supplied to heifers were restricted based on DMI, heifers among treatments were essentially fed different diets, as CP and energy level was altered. Therefore, as energy dense diets were provided, it was expected that no effect of limit-feeding would be detected on growth. In literature, limit-feeding has been proposed as a method to control energy

intake, however, if rations provided are based on the “golden rule” of balanced rations, there is no practical reason for energy control. Moreover, even if it is presented as a technique to reduce feeding costs, practically energy dense diets may result in being more expensive, as a diet with a level of 2.55 Mcal of ME/kg compared to a diet providing 2.43 Mcal of ME/kg, cost € 203/t and € 190/t, respectively (Bach et al., 2021).

As a method to balance energy in heifer rations, inclusion of high fiber forages of low quality in the diets is suggested (Erickson et al., 2020). However, dilution of diets with low quality forages may affect DMI, especially when high NDF roughages, such as straw are contained in the diet. Dietary NDF is negatively correlated with DMI (Arelovich et al., 2008), with its provision being suggested at around 0.86-1% of BW (Hoffman et al., 2008; Su et al., 2017). The suppression of DMI in late gestation stage, is a common phenomenon among multiparous and primiparous cows (Grummer et al., 2004). Except the dietary NDF level, management practices, such as housing cows and heifers at the same pen during late gestation, has been found to further decrease DMI, as a result of social interactions (Neave et al., 2017).

As heifers have requirements for the following milk production and growth, a reduction of DMI might oppose a threat to energy balance and accelerate the incidences of metabolic diseases (Lacasse et al., 2018). Moreover, excessive fat deposition at calving, endanger the appearance of ketosis (Van Amburgh et al., 2019), hence a BCS of 3.5 is suggested as target BCS at calving (NASEM, 2021). It is reported that obese heifers have lower ease at calving and need 86% more assistance at parturition than thinner heifers (Funston et al., 2012). If overconditioning is observed around time of

calving, it is rather a carryover effect of the rapid growth gains during prepubertal period, than increased energy intake after puberty (Van Amburgh et al., 2019). Hence, during late gestation period, a further suppression of DMI due to management and nutritional practices should be avoided, while heifers should maintain the proper body condition so that their health status and productive performance will not be compromised.

Overall, nutritional and growth management of heifers from weaning to calving appears to play an important role on future performance. Early life programming and an accelerated plan of nutrition in the preweaning period, appear to pave the way of production levels of primiparous cows. In the prepubertal period attainment of proper BW at the first service, determines AFC at a farm level. Moreover, maintaining increased growth levels without excessive fat deposition, along with AFC determine first lactation and lifetime milk production levels. Last but not least, adequate growth levels to support ongoing growth of heifer and fetus developmental requirements secure a successive first lactation period, without compromising heifer health. Therefore, it is obvious that the quality of nutritional management during this 2-year rearing process, is a major factor affecting herd's future performance and thus farm's economic sustainability.

1.5. Environmental challenges for dairy cattle farming

1.5.1. Contribution of dairy cattle farming to GHG emissions, air quality and reactive N pollution

On the onset of the 20th century, global warming appeared as a potential future threat and more of a concern than a real problem (Weart, 2008). Nowadays, the consequences of global warming are more evident than ever.

Carbon dioxide (CO₂), nitrous oxide (N₂O), methane (CH₄) and fluorinated gases are greenhouse gases (GHG) and together with water vapor absorb the energy radiating from Earth's surface that otherwise would escape from atmosphere, creating a blanket-like layer and making conditions on Earth's surface warmer and inhabitable (EPA, 2024). However, the GHG emissions have been increased by human activities leading to a rise on land and water surface temperature from by 1° C during the decades from 1850 to 2020 (EPA, 2024).

The global warming potential (GWP) of each gas has been developed to allow comparisons of different gases impact and GWP-weighted emissions are usually expressed on a CO₂ equivalent (eqCO₂) basis (EPA, 2024). Even though CO₂ emissions are highly important, CH₄ and N₂O have 28 and 265 times higher GWP, respectively, than CO₂ in a period of 100 years (EPA, 2024). Additionally, assuming the same GWP, GHG with higher atmospheric lifetime, exhibit higher degree of warming (Amoo and Fagbenle, 2020). For CH₄ atmospheric life is considerably shorter than CO₂ (12.4 years in a 100 year lifespan; EPA, 2024) making it a target gas, when it comes to strategy implementation and short term gains towards global warming mitigation (Beauchemin et al., 2020).

In 2019, CH₄ and N₂O emissions accounted for 18% (11.0 ± 3.2 Gt eqCO₂) and 4 % (2.7 ± 1.6 Gt eqCO₂) of global net GHG emissions, respectively (Dhakal et al., 2022). Regarding CO₂ emissions, fuel combustion and industry contributed the majority of CO₂ emissions accounting for 64% of global net GHG emissions (38.0 ± 3.0 Gt eqCO₂), while CO₂ emissions from Land Use, Land-Use Change and Forestry (LULUCF) were considerably lower at 11% of global

net GHG emissions ($6.6 \pm 4.6 \text{ Gt}_{\text{eqCO}_2}$) (Dhakal et al., 2022). Worldwide, energy sector contributes the most at 34.0 % of total GHG emissions, whereas the sector of agriculture, forestry and other land use (AFOLU) is responsible for 22.0 % of total anthropogenic GHG emissions (Figure 1.7; Dhakal et al., 2022; Nabuurs et al., 2023).

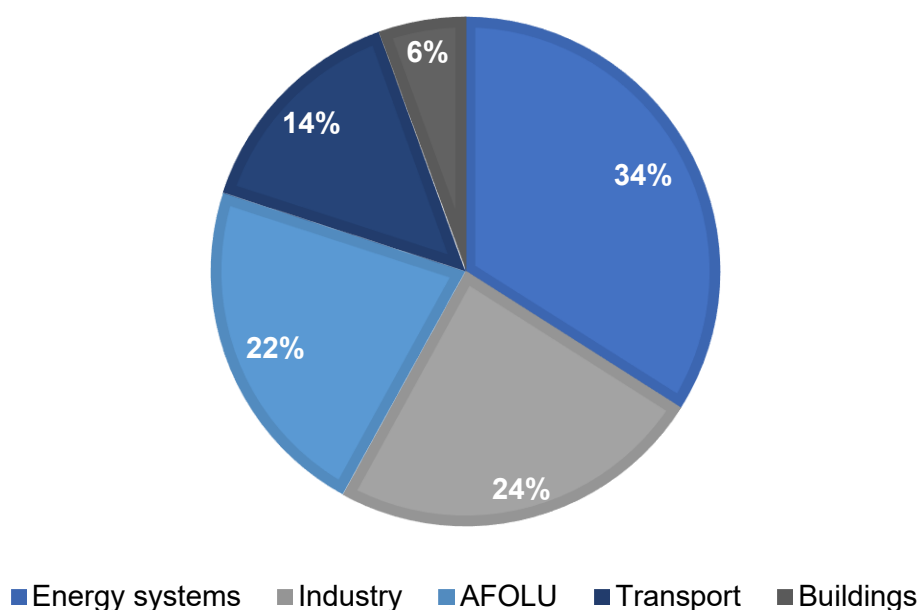


Figure 1.7. Contribution of different sectors to total net anthropogenic GHG emissions, where AFOLU: Agriculture, Forestry and Other Land Use (Dhakal et al., 2022).

Based on IPCC (1996) guidelines, AFOLU sector is divided into: a) net anthropogenic emissions from LULUCF, which represent harvested wood products and any change in land coverage and management, and b) net emissions from agriculture, containing the following subcategories: livestock (enteric fermentation, manure management), managed soils (biomass burning, liming, urea application, rice cultivation and direct and indirect emissions from

managed soils). Within AFOLU sector, LULUCF and agriculture account for 51.0 % and 49.0 % of global AFOLU eqCO_2 emissions, respectively, while LULUCF and agriculture individually contribute 11.2 % and 10.8 % of total GHG eqCO_2 emissions (Figure 1.8; Nabuurs et al., 2023).

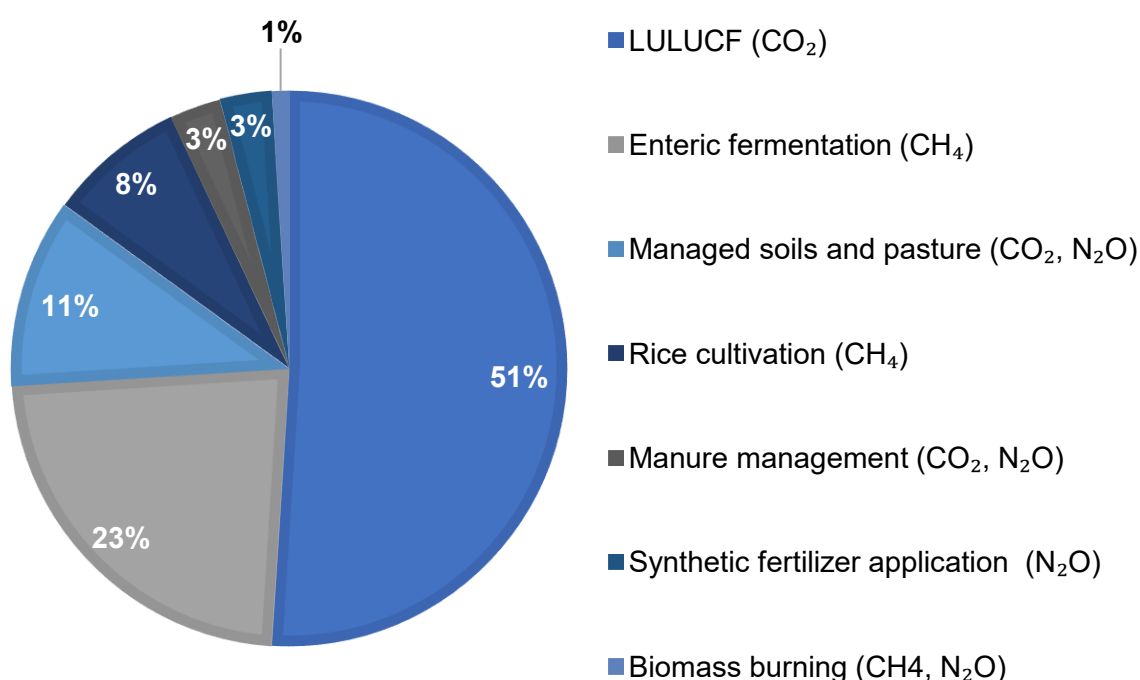


Figure 1.8. Contribution of subsectors to GHG emissions from Agriculture, Forestry and Other Land Use sector, where LULUCF: Land Use, Land Use Change and Forestry (adapted from Nabuurs et al. (2023))

For the period from 2010-2019, when examining net anthropogenic emissions from AFOLU by gas, CO_2 emissions were the highest and accounted for $5.9 \pm 4.1 \text{ Gt}_{\text{eqCO}_2} \text{ yr}^{-1}$, while CH_4 and N_2O were lower at 4.2 ± 1.3 and $1.8 \pm 1.1 \text{ Gt}_{\text{eqCO}_2} \text{ yr}^{-1}$ (Table 1.4; Nabuurs et al., 2023). It is noted that, the CO_2

emissions include emissions from land-use change (deforestation, afforestation etc.), wood harvest, land management and urea application.

Table 1.4. Net anthropogenic emissions from Agriculture, Forestry and Other Land Use (AFOLU) and contribution to total net anthropogenic emissions (annual averages for 2010-2019; adapted from Nabuurs et al. (2023)).

	AFOLU emissions	Non- AFOLU emissions	Total emissions	AFOLU % of total emissions
CO ₂ , Gt _{eq} CO ₂ yr ⁻¹	5.9 ± 4.1	36.2 ± 2.9	42.0 ± 29.0	14 %
CH ₄ , Gt _{eq} CO ₂ yr ⁻¹	4.2 ± 1.3	5.9 ± 1.8	10.2 ± 3.0	41 %
N ₂ O, Gt _{eq} CO ₂ yr ⁻¹	1.8 ± 1.1	0.8 ± 0.5	2.6 ± 1.5	69 %
Total	11.9 ± 4.4	44.0 ± 3.4	55.9 ± 6.1	22 %

Respectively in the U.S., agricultural sector itself accounts for 9.4 % of total gross GHG emissions, with energy sector accounting for 82.0 % of total gross GHG emissions (EPA, 2024). As one of the main rumen fermentation end products of carbohydrates is CH₄ (Beauchemin et al., 2020), enteric CH₄ production is responsible for the 32.5 % of total agricultural _{eq}CO₂ emissions (EPA, 2024). In total, CH₄ deriving from enteric fermentation and manure management is responsible for 3.0 % and 1.0 % of total gross GHG emissions (EPA, 2024). As shown in Table 1.5, enteric methane emissions from beef and dairy cattle account for 71.1 % and 25.4 % of agricultural _{eq}CO₂ emissions, respectively, consisting 2.9 % of total GHG emissions (EPA, 2024).

Table 1.5. Contribution of different livestock animal enteric methane emissions to agricultural eqCO_2 and total GHG emissions.

Animal	% of agricultural eqCO_2 emissions	% of total GHG emissions
Beef cattle	71.13	2.16
Dairy cattle	25.39	0.77
Swine	1.61	0.05
Horses	0.52	0.02
Sheep	0.67	0.02
Goats	0.36	0.01
American Bison	0.26	0.01
Mules and asses	0.05	<0.01

In addition, CH_4 and N_2O deriving from the decomposition of manure under anaerobic conditions during storage contributes 13.8 % to total agricultural eqCO_2 GHG emissions and 1.3 % of total GHG emissions. As shown in Table 1.6, beef and dairy cattle accounting for 0.7 % and 5.6 % of CH_4 emissions, while concerning N_2O emissions they are responsible for 1.1 % and 1.0 % of total emissions deriving from manure management, respectively (EPA, 2024). Consequently, within agricultural sector, enteric fermentation and manure management are of a main concern that livestock sector needs to deal with. However, even though the contribution of beef and dairy cattle in GHG emissions is not negligible, it is considered rather low compared to other non-livestock sectors.

Table 1.6. Contribution of different livestock animal methane (CH₄) and nitrous oxide (N₂O) emissions deriving from manure management to agricultural eqCO₂ and total GHG emissions.

Animal	% agricultural eqCO ₂ emissions	% of total GHG emissions
CH₄		
Dairy cattle	5.6	0.5
Swine	4.0	0.4
Poultry	0.5	<0.1
Beef cattle	0.7	0.1
Horses	<0.1	<0.1
Sheep	<0.1	<0.1
N₂O		
Beef cattle	1.1	0.1
Dairy cattle	1.0	0.1
Swine	0.3	<0.1
Poultry	0.4	<0.1
Sheep	0.1	<0.1
Horses	<0.1	<0.1
Total	13.8	1.3

Besides the increased concerns about GHG emissions, air quality and air pollution are a problem for many countries. Air pollution has been responsible for human and animal health problems, such as respiratory and cardiovascular diseases or premature mortality (Cambra-López et al., 2010;

Kim et al., 2015). Emissions from animal production systems contribute in the degradation of air quality. Main air pollutants are considered the carbon monoxide (CO), nitrogen dioxide (NO₂), tropospheric ozone (O₃), sulfur dioxide (SO₂) and the airborne particulate matter (PM; Place and Mitloehner, 2010). In dairy cattle farming, PM derives from feed harvesting and dust from open lot dairy farms, volatile organic compounds (VOC), emitted mainly from silage, and ammonia (NH₃) emissions (Place and Mitloehner, 2010). Moreover, a combination of pollutants may bring further increase in PM concentration, as in the case of VOC and NH₃ reaction with nitrogen oxides (NO_x) present in atmosphere (Place and Mitloehner, 2010; Sutton et al., 2011). Based on diameter of PM, PM_{2.5} and PM₁₀ have been identified, which include particles of less than 2.5 µm and 10 µm, respectively (European Environment Agency, 2022). Ammonia serves as a precursor to PM_{2.5} that is considered more harmful than PM₁₀, and has been accused for increasing the risk of premature human deaths (Wyer et al., 2022). Agriculture accounts for 3.0 % of total PM_{2.5} and 16.0 % of total PM₁₀ emissions (EEA, 2022), while in the agricultural sector, 14.0 % of NH₃ emissions deriving from cattle contribute to PM_{2.5} fraction (Wyer et al., 2022).

Except for the fact that NH₃ has the greatest impact in the formation of PM (Brunekreef et al., 2015), it is also a reactive form of N (N_r). In natural environment, non-reactive nitrogen (N₂) in its atmospheric form was converted to N_r through biological fixation, biomass burning and lightning (Erisman et al., 2013). However, the availability of N_r was limited and humanity, based on Haber-Bosch process, managed to produce NH₃ and develop synthetic fertilizers to increase crop productivity (Sutton et al., 2011). Agriculture, fossil

fuel combustion and industrial production were highly depended on N_2 fixation to produce various forms of N_r , that include inorganic forms such as NH_3 , N_2O , nitrogen oxides (NO_x), ammonium (NH_4^+), nitrates (NO_3^-), and organic compounds such as proteins, amines and urea (Galloway et al., 2003; Fowler et al., 2013). The utilization of N_r benefited society and industry, contributing to the population increase and life quality improvement (Erisman et al., 2013).

In agricultural systems and under natural conditions N_r will be immobilized or will be recycled back as N_2 through denitrification process (Sutton et al., 2011). In Figure 1.9, blue and other color arrows are depicting the intended and unintended flows of N_r , respectively, while orange and blue boxes are illustrating the main N_r pollutants and environmental consequences, respectively (Sutton et al., 2011).

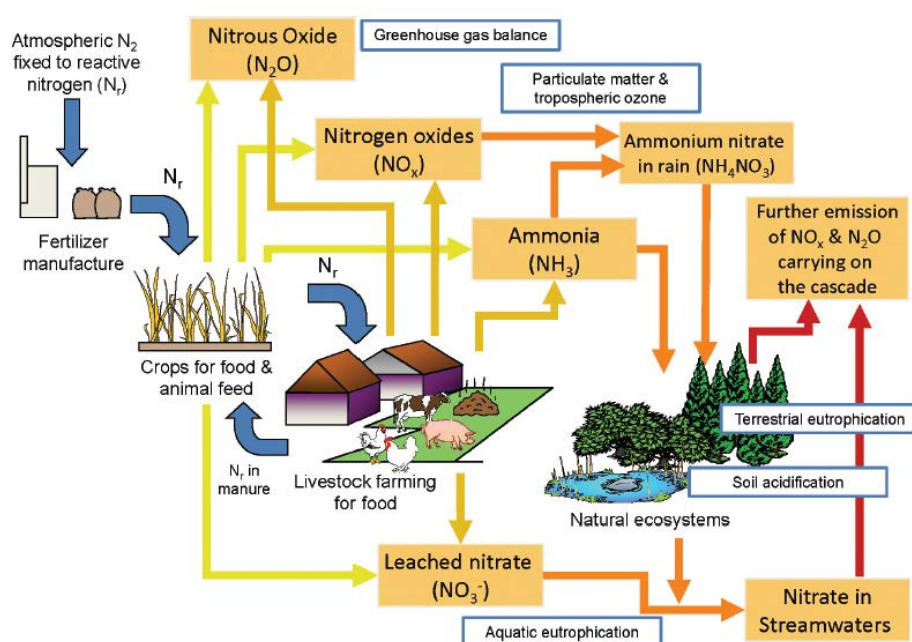


Figure 1.9. Summary of the nitrogen cascade in which intended (blue arrows) and unintended (rest arrows) flows of N_r are illustrated. Main N_r pollutants (orange boxes) and environmental consequences are depicted (blue boxes; Sutton et al., 2011).

As it is shown, N_r contained in fertilizers is applied on fields for crop cultivation intended to be used as animal feed and food (Figure 1.9). When a system is under a state of balance, N_r contained in the manure will be deposited as natural fertilizer on fields and finally N_r will be utilized by plants for their growth requirements (Sutton et al., 2011). However, the rate of denitrification is lower than the accumulation and creation of N_r in the systems (Galloway et al., 2003). The extended use of N_r through the synthetic fertilizer application and combustion for energy supply and transportation purposes caused additional unintended N_r flows (orange arrows, Figure 1.9) disrupting the natural denitrification back to N_2 (Galloway et al., 2003; Sutton et al., 2011). The conversions and transfers of N_r through atmospheric, aquatic and terrestrial systems include N_r leakages to ground water or atmosphere (Fowler et al., 2013) and can trigger negative sequences that are described by nitrogen cascade (Figure 1.9; (Galloway et al., 2003). The main N_r pollutants are responsible for disruption of greenhouse balance, eutrophication, aquatic ecosystems and soil acidification, soil's organic content and biodiversity alteration and ozone depletion (Sutton et al., 2011; Erisman et al., 2018).

In the EU, agriculture is the main sector emitting NH_3 in the air, through practices such as the use of synthetic fertilizers to improve feed and food cultivation, manure management and application on fields and accounts for the 94.0 % of the total NH_3 emissions (Figure 1.10; (Malherbe et al., 2022)). Livestock sector activities, such as manure management, manure application to fields and grazing, account for the 72.0 % of agricultural NH_3 emissions (Malherbe et al., 2022). Among different type of animals, cattle contribute the

most to NH_3 emissions, with a percentage of 51.3 % of total NH_3 emissions in EU, followed by swine at 27.5 % (Malherbe et al., 2022).

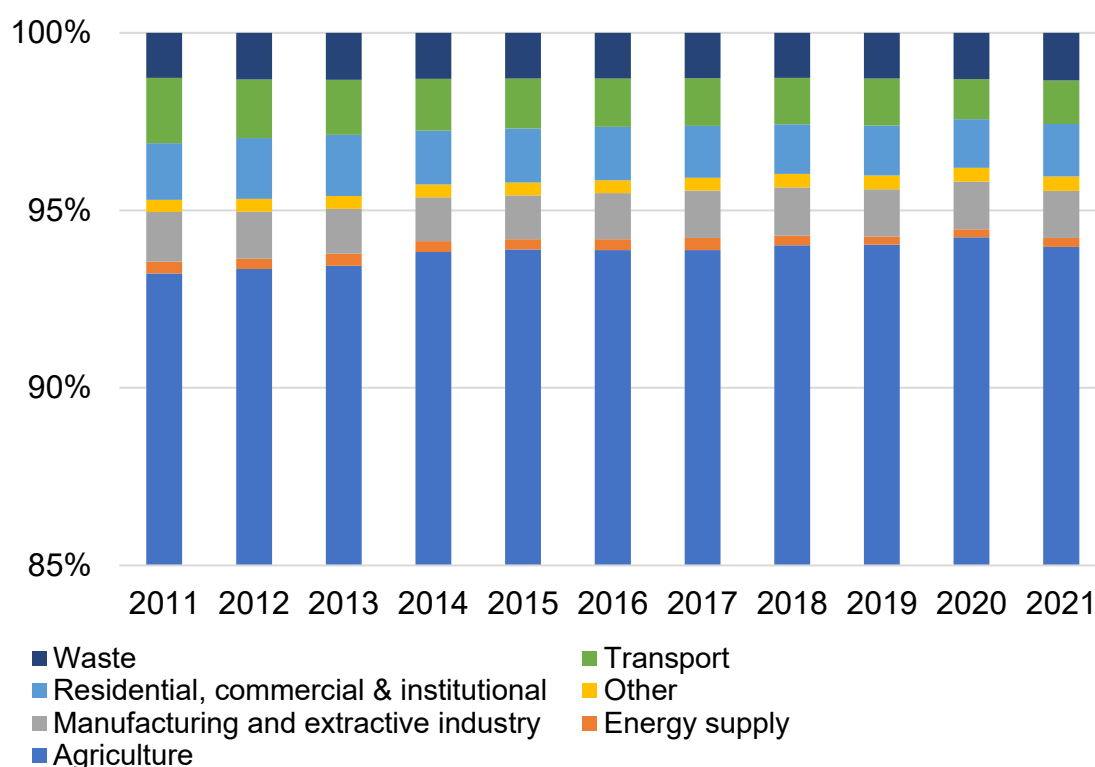


Figure 1.10. Trends of ammonia emissions in the last decade, with agriculture being the main ammonia emitter (adapted from data viewer; (EEA, 2023)).

Dairy cattle farming has been linked with N_r pollution. Emissions of NH_3 , N_2O and NO_x in the atmosphere and leaching of NO_3^- and NH_4^+ to ground and surface water are occurring through crop and manure management practices (Dijkstra et al., 2013; Dijkstra et al., 2018). As shown in Figure 1.11, cattle manure is applied on fields either as stored manure collected from farm storage units or while animals are grazing (Jensen et al., 2011; Bougouin et al., 2016).

The processes of NH_3 formation and volatilization starts immediately with the urine and feces excretion (Hristov et al., 2011). Urea, which is contained in urine at a percent of 50-90 % (Bussink and Oenema, 1998), is rapidly hydrolyzed by urease, which is an enzyme produced by rumen microbes and excreted in feces (Hristov et al., 2011). As a result, NH_3 and NH_4^+ is formed (Bougouin et al., 2022), with 4-41 % of N present in urine being volatilized, while only 1-13 % of N present in feces will be volatilized and lost (Bussink and Oenema, 1998).

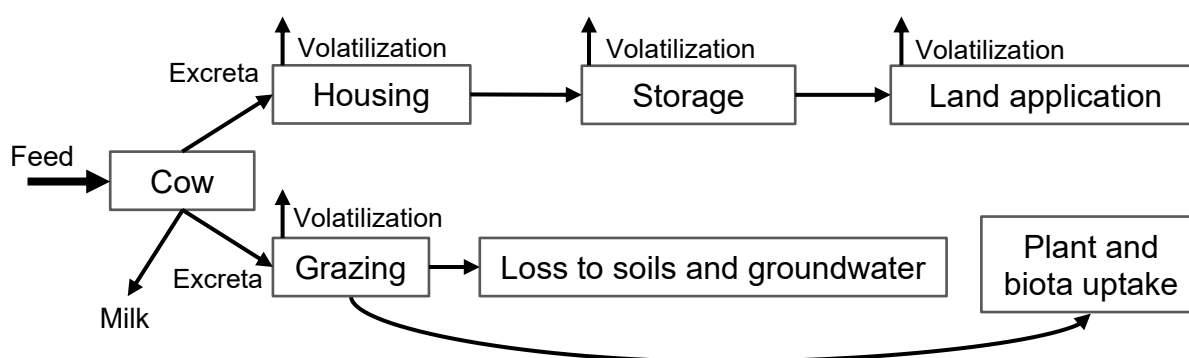


Figure 1.11. Pathway of nitrogen flow beginning from animal consumption to excretion and deposition on the environment (Bougouin et al., 2016).

The volatilization rate highly depends on NH_3 contained in the manure, with N contained in urine being mainly responsible, as N found in feces is mainly organic bound and at a lower extend volatile (Hristov et al., 2011; Behera et al., 2013). The N loss in the form of NH_3 accounts for the 25-50% of manure N excretion (Hristov et al., 2011). Emissions of NH_3 deriving from manure range from 6.7-18.2 g/d per cow and 12.2-25.4 g/d per heifer (Powell et al., 2008a; Powell et al., 2008b). James et al. (1997) reported NH_3 emissions of 59 g/d per cow, with a SD of 65 g/d per cow. A metanalysis conducted by Bougouin et al.

(2016) reported average NH_3 emissions of 59.3 g/d per cow, ranging from 5.0 to 304 g/d per cow ($\text{SD}=49.3$). These findings point out that there is a high variation regarding NH_3 emission estimates. Various factors affect this loss such as manure handling system, housing and bedding type, ventilation, environmental temperature, season and nutrition (Powell et al., 2008a). The CP content of the diet is associated with the amount of N contained in manure (Agle et al., 2010). Hence, a potential decrease of N losses through NH_3 volatilization can be realized by decreasing the provided CP of the diets (Agle et al., 2010). Frank and Swensson (2002) found that when dietary CP level was reduced from 17.0 % DM to 13.5% DM, a reduction of 45 % in NH_3 emissions from dairy cattle was detected. Bougouin et al. (2016) conducted a metanalysis and found that on average 9.3 % of N intake was lost as NH_3 (ranging 1.6-30.0 %, $\text{SD}=6.6$), whereas NH_3 emissions were moderately affected by N consumption ($r=0.55$) and CP level ($r=0.51$). Moreover, in the mixed effects model used milk yield, DMI and CP level explained the 45.5 % of heterogeneity, indicating positive effects of CP content and DMI on NH_3 emissions (Bougouin et al., 2016).

In conclusion, although N is essential for sustaining life and production systems, the reactive forms tradeoffs among atmospheric, aquatic and terrestrial ecosystems impact negatively environmental balance and create various pollution phenomena. The so-far implemented practices by animal production systems and especially, dairy cattle, contributed to N_r pollution through fertilizer, manure application, and manure management. The effects of diet and especially CP level on NH_3 emissions indicate that the adjustment of diets can be a critical step towards management of N losses.

1.5.2. Factors affecting Lifetime Nitrogen Use Efficiency of dairy farms and the role of dairy replacement heifers

The increase of NUE has been suggested as system related action to mitigate reactive N pollution (Sutton et al., 2011). As, the NUE is practically a ratio ($\text{N output of a system} / \text{input N into the system}$; (Erisman et al., 2018), an increase is possible either through an increase of the output N or decrease of the input N into a system. At a cow level a measurement of NUE is the conversion of dietary N into milk N, and it is defined as milk N use efficiency (MNE; $\text{g of milk N content} / \text{g of dietary N}$; Calsamiglia et al., 2010). In dairy cows N utilization is relatively low, with MNE presenting high variation and ranging from 14.0 to 45.3 % (Huhtanen and Hristov, 2009). An increase in MNE can be achieved through the adaptation of N intake at lower levels or an increase in N contained in milk. It is noted that a positive correlation between milk protein content and milk yield has been detected (Bougouin et al., 2016). Thus, an increase in milk production provided that N intake does not increase may further improve MNE (Huhtanen and Hristov, 2009).

At a farm level, MNE appears to be among the factors that highly impact lifetime nitrogen use efficiency (LNE) of the farm (Foskolos and Moorby, 2018). Besides MNE, farm LNE is affected by calving interval (CI), a reproduction parameter. Even though the extension of CI has been reported to benefit herd profitability (Arbel et al., 2001), it has been found that cows with lower voluntary waiting period, thus shorter CI, had higher fat and protein corrected milk yield per day of CI (Burgers et al., 2021). Furthermore, Foskolos and Moorby (2018) found a negative correlation between NUE of lactation and LNE, meaning that

extended CI can bring a reduction in NUE not only during lactation period, but also through cow's lifetime.

In the same study, another factor that was identified to affect farm overall LNE was the AFC. It was found a negative correlation among AFC, LNE and replacement NUE (Foskolos and Moorby, 2018). The extension of non-productive period which results in higher AFC, highly affects the total N requirements for heifer growth, meaning that higher N losses can occur, leading to a further increase in total NH_3 emissions and, thus, to a reduction of farm LNE (Foskolos and Moorby, 2018). The intensity under which heifers are growing after weaning directly affects the onset of breeding process and ultimately the AFC (Schillo et al., 1992; Funston et al., 2012; Heinrichs et al., 2017). Moreover, the dietary N intake is considered the main factor affecting N excretion (Dijkstra et al., 2013). Foskolos and Moorby (2018) found that MP:CP ratio was the factor with the highest impact on replacement NUE. Therefore, other than reproduction parameters, nutritional management of replacement heifers, and especially N supply, appears to affect farm LNE.

Overall, understanding and controlling the factors that impact N losses is a crucial step towards improvement of overall farm LNE. Heifers comprise a major part of dairy herds. Better heifer growth and conception rates may improve AFC, shortening the non-productive period and minimize N losses. Hence, proper nutrition and reproduction management of heifers may serve as a tool to mitigate and improve overall farm LNE.

1.5.3. Increasing Nitrogen Use Efficiency of dairy replacement heifers

Understanding the mechanisms behind protein and carbohydrate utilization and metabolism, is crucial step towards the increasing NUE of growing heifers. Growing replacement heifers have protein requirements for growth, maintenance and pregnancy, with the required amount of protein depending on growth stage (NASEM, 2021). Dietary protein, and especially the rumen degradable protein (RDP) is used to provide true protein (TP) and non-protein N (NPN), which are converted to rumen NH_3 and depending on carbohydrate availability, both are used for microbial protein synthesis (Bach et al., 2005). Microbial protein in combination with the rumen undegradable protein (RUP) provide additional amino acids (AA) that are later ingested and absorbed in the small intestine, with microbial protein accounting for 50–80% of the total protein absorbed (Storm and Orskov, 1983; Huhtanen and Ahvenjärvi, 2022). The AA reaching the intestine consist the MP provided to the animal and are used for tissue deposition and growth (Huhtanen and Ahvenjärvi, 2022).

When excessive rumen NH_3 is accumulated, the surplus is transported to the liver and converted to urea (Bach et al., 2005). The amount of N consumed by the animal and not partitioned into milk or body protein accretion will be excreted in feces and urine (Lapierre et al., 2018). In contrast, when dietary protein is limited the urea transported in the liver will be recycled back to the rumen, through rumen epithelium or salivary secretions, to maintain a positive N balance (Wickersham et al., 2009; Batista et al., 2017). In growing heifers urea recycling appears to be driven by N intake, with heifers consuming less N incorporating more N into microbial protein (Marini and Van Amburgh,

2003). Specifically, heifers used 43.0, 25.0 and 6.0% N recycled for microbial protein synthesis when consumed diets containing CP levels of 9.1, 15.6 and 21.3% DM, respectively (Marini and Van Amburgh, 2003). Ruminants can survive solely depended on NPN sources (Van Soest, 1994), however, growth cannot be exclusively supported by microbial protein synthesis (Merchen and Titgemeyer, 1992), meaning that RUP is essential to meet AA demands of a growing heifer (Lascano et al., 2016). Of course, as a first step in an effort to maximize ruminal NUE, is to minimize N losses in NH_3 form.

In this direction, synchronizing energy and protein supplied to rumen microorganisms has been suggested as a method to enhance microbial growth and, therefore, improve nutrient utilization (Hoover and Stokes, 1991; Firkins, 1996). Through carbohydrate fermentation, degradation to simple sugars (glucose, hexoses and pentoses) and, finally, glycolysis, adenosine triphosphate (ATP) is generated and used for microbial growth (Hoover and Stokes, 1991), while rumen microorganisms produce volatile fatty acids (VFA; mainly acetate, propionate and butyrate), which later become an energy source for the heifer. Furthermore, based on the Cornell Net Carbohydrate and Protein System (CNCPS), structural carbohydrate-fermenting bacteria require mainly NH_3 , and non-structural carbohydrate-fermenting bacteria require mainly AA and less NH_3 to meet growth demands (Russell et al., 1992), suggesting that protein degradation takes place first to release these AA. However, ruminal degradation of different protein and carbohydrate fractions occurs at different rates depending on the source, with each fraction exhibiting different passage rates from the rumen as well (Higgs et al., 2015). For example, soluble proteins (SP) and sugars are rapidly available in the rumen, while proteins bound to NDF

(NDIP) and NDF are slowly degraded in the rumen. Thus, carbohydrate and protein fractions supplied to the rumen determine the amount of energy and protein available for microbial protein synthesis. For maximizing microbial protein synthesis, it is important to meet microorganisms' growth requirements and ensure the synchronous release of available energy and NH_3 in the rumen.

Mechanistic models may be valuable tools for assisting with nutrient synchrony, especially because they contain functions to balance for microbial protein synthesis, considering both energy and protein availability (e.g., the rumen NH_3 balance in CNCPS). These models are not dynamic, considering a static daily supply of nutrients in one meal (Van Amburgh et al., 2015; NASEM, 2021). However, heifers have demonstrated a feeding pattern with several meals taken within a day (Robles et al., 2007; Green et al., 2013). Perhaps this may explain why the CNCPS underestimates rumen microbial N outflow and overestimates rumen feed N outflow, though it still reaches an accurate rumen nonammonia N outflow prediction (Van Amburgh et al., 2015). Ongoing efforts to develop the CNCPS in a dynamic platform (version 7) may further improve its accuracy and precision and thus assist in formulating diets with optimal energy and protein synchrony. In this direction, the accurate prediction of protein requirements and thus precise protein feeding is of high importance when it comes to minimizing N losses (Hristov et al., 2011). An important step was the shift between CP-based nutritional systems and digestible CP systems that take into consideration MP requirements, RDP requirements and intestinal AA absorption (Tedeschi et al., 2015; Lapierre et al., 2018; Huhtanen and Ahvenjärvi, 2022). The recent recommended protein requirements based on the NASEM (2021) of 224 and 420 kg replacement heifers, gaining 0.84 kg/d

and a MBW of 700 kg, are 610 and 772 g/d MP, respectively. An available nutritional model that balances rations based on MP is the mechanistic CNCPS. Van Amburgh et al. (1998a) evaluated the prediction accuracy of the CNCPS (version 3) and NRC (1989) for the nutrient requirements of growing heifers and demonstrated that the CNCPS was more accurate for estimating MP requirements ($R^2 = 0.76$ compared with 0.68 for the CNCPS and NRC, respectively). Later, Fox et al. (1999), based on the Beef NRC (1996), refined the heifer body composition and nutrient requirement equations, which were then adopted by the NRC (2001). Currently, the CNCPS for growth is based on the Beef NRC (1996), which is the same as the NRC (2001). However, the CNCPS is considered a diet formulation model with high sensitivity, especially in low-protein diets. In a dataset with a mean milk yield of 34.6 kg/d, when MP was the first limiting factor (relatively low supply of N), the updated version of CNCPS, version 6.5, predicted the MP-allowable milk and first limiting nutrient with errors of 1.1 kg and 1.6 kg of milk, respectively (Van Amburgh et al., 2015).

Overall, understanding N metabolism can define the extent to which manipulation of the diet composition can intervene with the aim to affect N utilization, while the use of mechanistic nutritional models proven sensitive to low-protein diets may be a valuable tool to further minimize N losses.

1.5.3.1. Effects of crude protein level on nitrogen use efficiency of replacement heifers

Towards the direction of decreasing N losses, an increase in replacement NUE can be achieved through the reduction of N intake without impacting growth. Taking into consideration the mechanisms of N utilization by the animal there are certain dietary alterations that can be followed to decrease

N intake, while increase N utilization. Therefore, reducing the CP level of replacement heifers' diets might as well, decrease N excretion and improve NUE. However, not only the CP level affects the NUE, but also changes in ratio between rumen degradable and undegradable protein may further improve N utilization and thus NUE (Davidson et al., 2003).

The NUE of replacement heifers depends on growth stage. Zanton and Heinrichs (2016) observed that even though N retention and digestibility were not different between younger heifers and older heifers that were fed high- and low-energy density diets, the younger heifers retained higher levels of N when N retention was expressed as a proportion of consumed or digested N. They attributed the higher N utilization to better post-absorptive metabolism. However, it is well established that in the period from birth until weaning, there is higher body protein accretion than in other phases (Van Amburgh et al., 2019). Moreover, in Figure 1.5 it is shown that between the period from birth until weaning, there is higher body protein accretion than other phases. Therefore, birth until weaning period is expected to be the most efficient one. Chapman et al. (2017) provided milk replacers containing two different levels of CP at 20 % DM and 26 % DM to 3 groups of calves. To all groups a starter containing 20.7 % CP of DM was offered ad libitum. Calves of conventional treatment were fed 446 g of DM of 20 % CP milk replacer, calves of moderate and aggressive treatment were fed 669 g and 892 g of DM of 26 % CP milk replacer, respectively. The NUE ranged from 44.4 to 52.7 %, with calves in conventional treatment exhibiting the higher efficiency. No difference was observed between the rest of groups (46.7 and 44.4 %, for moderate and

aggressive treatment, respectively), meaning that the increase of CP supply decreased the NUE.

After weaning and until the first calving, it is expected that NUE will be lower compared to the first stage of life, as N retention is reduced while heifer reaches maturity (Lobley, 1993). Several studies have examined the appropriate level of CP supply, however the findings about N retention appear to highly vary. Marini and Van Amburgh (2003) fed 265 kg heifers diets containing CP at 9.0, 11.8, 15.6, 18.6 and 21.2 % DM at a forage to concentrate ratio (F:C) 30:70 %. They found that N balance, N recycled and microbial protein was better for heifers fed diets at 9.0 and 11.8 % of DM, leading to NUE of 22.37 % in both cases compared to 20.0, 18.1 and 15.9 % for 15.6, 18.6 and 21.2 % CP levels of DM, respectively. Hoffman et al. (2001) offered heifers of 410 kg BW diets containing levels of CP at 8.0, 11.0, 13.0 and 15.0 % DM. Even though ADG did not differ among treatments, maximum N retention was observed at CP level of 13.0 % DM. In the same study, it was calculated that the group fed the 13 % CP level exhibited the highest NUE at 13.7 %. Johansen et al. (2022) fed 340 and 435 kg heifers (12 and 16 months old, respectively) 3 levels of CP at 12.2, 14.6 and 17.0 % DM and 9.8, 12.2 and 14.6 % DM, respectively. Based on their data we calculated that the highest N retention for 12 months heifers was found in diets containing CP of 12.2 % DM, with NUE at 15.6 %, while at the same CP level, NUE of 16 months heifers was 8.5 %. When the diet with CP level at 17.0 % DM was fed to 12 months heifers, NUE decreased at 3.5 %. Adachi et al. (2006) fed heifers at 9 weeks prepartum, diets containing a low and a high level of CP at 11.5 and 14.1 % DM, respectively. It was calculated that NUE was 22.1 and 36.9 % for low and high group,

respectively. In contrast, Johansen et al. (2022) fed 20 months heifers at 161 d of gestation, diets with CP at 9.8, 12.2 and 14.6 % DM, resulting in NUE of 14.7, 11.5 and 7.0 %, respectively. Moreover, limiting feeding of heifers has been suggested as a method to decrease the DM excreted and therefore, minimize the amount of nutrient losses. Hoffman et al. (2007) limit fed pregnant heifers of 17.5 months of age with diets containing 11.3 %, 12.7 % and 14.2 % CP of DM and DMI set at 100, 90 and 80 % of the control diet, respectively. They found that heifers consuming 80 and 90 % of the control diet, had lower N retention. Moreover, no difference was observed on N utilization and even though reduced DM excretion was observed in limit fed heifers, the N excretion and NUE was not improved, resulting in NUE of 23.2, 21.8 and 19.4 % for heifers in groups of 100, 90 and 80 % of control DMI, respectively.

As a conclusion, it appears that N retention is maximized in diets containing rather low levels of CP, while diets with higher content in CP might not bring any further improvement in N utilization.

1.5.3.2. Effects of rumen degradable and undegradable protein on nitrogen use efficiency of replacement heifers

Another strategy to reduce NH_3 losses is the alteration of protein degradability in the rumen (Tamminga, 1996; Davidson et al., 2003). Once the microbial requirements for growth have been met from RDP sources, RUP may further improve N retention (Tomlinson et al., 1997). Silva et al. (2018) examined the effects of RUP levels on N utilization of 276 kg replacement heifers. Diets contained levels of RUP at 38.9, 44.1, 48.9 and 54.0 % CP, while CP levels were similar among treatments at 15.14, 15.20, 15.26 and 15.32 % DM, respectively. Nitrogen balance was found to be lower for highest RUP

levels at 48.9 and 54.0 % CP diets, with N intake and N fecal excretion not being affected by RUP level. A tendency for reduction of urinary N was observed with increased level of RUP (97.3, 97.4, 89.7 and 85.5, for RUP at 38.9, 44.1, 48.9 and 54.0 % CP, respectively). Moreover, a tendency for increased N retention was observed at the higher levels of RUP, while NUE was for higher at 9.5, 10.6, 14.8 and 14.4 % (P-value =0.035) for the higher levels of RUP at 38.9, 44.1, 48.9 and 54.0 % CP, respectively. Therefore, increased levels of RUP at 48.9-54.0 % CP increased N retention and NUE, while N losses were decreased without affecting intakes. They concluded that the higher levels of RUP were similar and proposed that 48.9 % RUP of CP can be utilized in diets of growing heifers that need to maintain high growth rates (Silva et al., 2018).

In contrast, as a strategy to increase nutrient utilization Zanton et al. (2007) proposed the increase of soluble protein (SP) content (and therefore RDP content), when replacement heifers are fed diets relatively high in forage and thus high in NDF content. Zanton et al. (2007) examined the effects of SP and RUP levels of heifers fed high forage diets in a 2 x 2 factorial experimental design (Low SP treatment; Low RUP (RUP 38.1 % CP, SP 27.3 %CP) and High RUP (RUP 43.9 % CP, SP 26.6 % CP and High SP treatment; Low RUP (RUP 38.7 % CP, SP 35.6 %CP) and High RUP (RUP 46.7 % CP, SP 37.7 %CP)) and observed no effect on N utilization. Lascano et al. (2016) investigated the effect of high RDP levels on N utilization in diets of low or high forage content of precision fed heifers. Heifers of 15.5 months old were assigned to a 3 x 3 Latin square design and to two dietary treatments with different F:C ratio, low forage (LF) and a high forage (HF) at a F:C ratio of 45:55 and 90:10,

respectively, with 3 different proportion of forages at (a) 33 % wheat straw and grass hay and 67% corn silage; 33 % treatment, (b) 50 % wheat straw and grass hay and 50% corn silage; 50% treatment and (c) 67 % wheat straw and grass hay and 33 % corn silage; 67% treatment. The CP and RDP level of diets ranged between 10.5 - 11.4 % DM and 64.0 - 66.3 % CP for HF heifers and 12.2 - 12.7 % DM and 63.8 - 64.0 % CP for LF heifers, respectively. It was observed that N intake did not differ among treatments, total tract digestibility was higher for LF heifers, while N apparent digestibility was not different between F:C ratio treatments. Moreover, no difference was observed on urinary N excretion, however N retention was higher for all LF heifers, with heifers at the 33 % treatment exhibiting the highest retention at 69.4 g/d compared to 53.3 g/d for HF heifers of 33 % treatment. Based on these data, it was calculated that NUE was highest for the group of 33 % treatment of LF heifers at 37.6 %, while the lowest NUE had the 67 % inclusion of HF heifers at 14.6 %. The authors concluded that heifers of LF diets benefited the most of high RDP levels, while HF diets presented poor N and nutrient utilization, highlighting the importance of higher RUP levels with higher levels of forage inclusion under precision feeding conditions.

Overall, based on the previous studies it is observed that higher RUP levels benefit N utilization, while the increase of RDP levels of high forage diets did not bring any further improvement on N utilization. However, it is evident that during the period from weaning up to first calving there is a high variation regarding NUE in both cases of altering the CP and RUP levels. Therefore, the high variation that is observed on research results regarding replacement heifers indicates that there is opportunity for improvement.

1.6. Objectives

The aim of the current thesis was to improve the NUE of dairy replacement heifers. Therefore, the objectives of the thesis were (a) to investigate the level of NUE of the feed in dairy replacement heifers, (b) to identify nutritional factors that impact and improve NUE of dairy replacement heifers (c) to propose a guide and proper management schemes that will enable the implementation of practices to mitigate nitrogen pollution deriving from dairy cattle farming. Therefore, three different studies were performed with the respective aims:

- I. **First study** (Chapter 2): to investigate the management conditions and the current status of heifers' NUE reared under Mediterranean conditions.
- II. **Second study** (Chapter 3): to examine the effect of different CP and RDP levels of diets to NUE of dairy replacement heifers.
- III. **Third study** (Chapter 4): to structure and describe a scheme under a holistic approach of the nutritional management including the animals, feeding and farm management to increase farm NUE.

1.7. References

- Abeni, F., Petrera, F., Le Cozler, Y., 2019. Effects of feeding treatment on growth rates, metabolic profiles and age at puberty, and their relationships in dairy heifers. *Animal* 13, 1020-1029. doi: <https://doi.org/10.1017/S1751731118002422>.
- Adachi, N., Kusuvara, T., Nonaka, I., Terada, F., 2006. Effect of close-up dry period protein level on preparturient nitrogen balance and lactating performance of primigravid and multiparous holstein cows. *Asian-Australas Journal of Animal Science* 19, 831-836. doi: <https://doi.org/10.5713/ajas.2006.831>.
- Agle, M., Hristov, A.N., Zaman, S., Schneider, C., Ndegwa, P., Vaddella, V.K., 2010. The effects of ruminally degraded protein on rumen fermentation and ammonia losses from manure in dairy cows. *Journal of Dairy Science* 93, 1625-1637. doi: <https://doi.org/10.3168/jds.2009-2579>.
- AHDB, 2024. Agriculture and horticulture development board. Retrieved on 8th of July 2024 from <https://ahdb.org.uk/dairy/uk-and-eu-cow-numbers>.
- Alvåsen, K., Dohoo, I., Roth, A., Emanuelson, U., 2018. Farm characteristics and management routines related to cow longevity: A survey among swedish dairy farmers. *Acta Veterinaria Scandinavica* 60, 38. doi: <https://doi.org/10.1186/s13028-018-0390-8>.
- Amoo, L.M., Fagbenle, R.L., 2020. Advanced fluids – a review of nanofluid transport and its applications. In *Applications of heat, mass and fluid boundary layers* (ed. Fagbenle, R.O., Amoo, O.M., Aliu, S. and Falana, A.), Woodhead Publishing, p. 281-382. doi: <https://doi.org/10.1016/B978-0-12-817949-9.00020-7>.

- Arbel, R., Bigun, Y., Ezra, E., Sturman, H., Hojman, D., 2001. The effect of extended calving intervals in high-yielding lactating cows on milk production and profitability. *Journal of Dairy Science* 84, 600-608. doi: [https://doi.org/10.3168/jds.s0022-0302\(01\)74513-4](https://doi.org/10.3168/jds.s0022-0302(01)74513-4).
- Archbold, H., Shalloo, L., Kennedy, E., Pierce, K.M., Buckley, F., 2012. Influence of age, body weight and body condition score before mating start date on the pubertal rate of maiden holstein–friesian heifers and implications for subsequent cow performance and profitability. *Animal* 6, 1143-1151. doi: <https://doi.org/10.1017/S1751731111002692>.
- Arelovich, H.M., Abney, C.S., Vizcarra, J.A., Galyean, M.L., 2008. Effects of dietary neutral detergent fiber on intakes of dry matter and net energy by dairy and beef cattle: Analysis of published data. *The Professional Animal Scientist* 24, 375-383. doi: [https://doi.org/10.15232/S1080-7446\(15\)30882-2](https://doi.org/10.15232/S1080-7446(15)30882-2).
- Bach, A., Ahedo, J., 2008. Record keeping and economics of dairy heifers. *Veterinary Clinics of North America: Food Animal Practice* 24, 117-138. doi: <https://doi.org/10.1016/j.cvfa.2007.10.001>.
- Bach, A., Ahedo, J., Kertz, A., 2021. Invited review: Advances in efficiency of growing dairy replacements. *Applied Animal Science* 37, 404-417. doi: <https://doi.org/10.15232/aas.2021-02164>.
- Bach, A., Calsamiglia, S., Stern, M.D., 2005. Nitrogen metabolism in the rumen. *Journal of Dairy Science* 88, E9-E21. doi: [https://doi.org/10.3168/jds.S0022-0302\(05\)73133-7](https://doi.org/10.3168/jds.S0022-0302(05)73133-7).
- Barkema, H.W., von Keyserlingk, M.A.G., Kastelic, J.P., Lam, T.J.G.M., Luby, C., Roy, J.P., LeBlanc, S.J., Keefe, G.P., Kelton, D.F., 2015. Invited

- review: Changes in the dairy industry affecting dairy cattle health and welfare. *Journal of Dairy Science* 98, 7426-7445. doi: <https://doi.org/10.3168/jds.2015-9377>.
- Batista, E.D., Detmann, E., Valadares Filho, S.C., Titgemeyer, E.C., Valadares, R.F.D., 2017. The effect of cp concentration in the diet on urea kinetics and microbial usage of recycled urea in cattle: A meta-analysis. *Animal* 11, 1303-1311. doi: <https://doi.org/10.1017/S1751731116002822>.
- Bazeley, K.J., Barrett, D.C., Williams, P.D., Reyher, K.K., 2016. Measuring the growth rate of uk dairy heifers to improve future productivity. *The Veterinary Journal* 212, 9-14. doi: <https://doi.org/10.1016/j.tvjl.2015.10.043>.
- Beauchemin, K.A., Ungerfeld, E.M., Eckard, R.J., Wang, M., 2020. Review: Fifty years of research on rumen methanogenesis: Lessons learned and future challenges for mitigation. *Animal* 14, s2-s16. doi: <https://doi.org/10.1017/S1751731119003100>.
- Beharka, A.A., Nagaraja, T.G., Morrill, J.L., Kennedy, G.A., Klemm, R.D., 1998. Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. *Journal of Dairy Science* 81, 1946-1955. doi: [https://doi.org/10.3168/jds.S0022-0302\(98\)75768-6](https://doi.org/10.3168/jds.S0022-0302(98)75768-6).
- Behera, S.N., Sharma, M., Aneja, V.P., Balasubramanian, R., 2013. Ammonia in the atmosphere: A review on emission sources, atmospheric chemistry and deposition on terrestrial bodies. *Environmental Science and Pollution Research* 20, 8092-8131. doi: <https://doi.org/10.1007/s11356-013-2051-9>.

- Bell, A.W., Burhans, W.S., Overton, T.R., 2000. Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. *Proceedings of the Nutrition Society* 59, 119-126. doi: <https://doi.org/10.1017/S0029665100000148>.
- Bellingeri, A., Cabrera, V., Gallo, A., Liang, D., Masoero, F., 2019. A survey of dairy cattle management, crop planning, and forages cost of production in northern Italy. *Italian Journal of Animal Science* 18, 786-798. doi: <https://doi.org/10.1080/1828051X.2019.1580153>.
- Bérodier, M., Brochard, M., Boichard, D., Dezetter, C., Bareille, N., Ducrocq, V., 2019. Use of sexed semen and female genotyping affects genetic and economic outcomes of Montbéliarde dairy herds depending on the farming system considered. *Journal of Dairy Science* 102, 10073-10087. doi: <https://doi.org/10.3168/jds.2018-16041>.
- Bougouin, A., Hristov, A., Dijkstra, J., Aguerre, M.J., Ahvenjärvi, S., Arndt, C., Bannink, A., Bayat, A.R., Benchaar, C., Boland, T., Brown, W.E., Crompton, L.A., Dehareng, F., Dufrasne, I., Eugène, M., Froidmont, E., van Gastelen, S., Garnsworthy, P.C., Halmemies-Beauchet-Filleau, A., Herremans, S., Huhtanen, P., Johansen, M., Kidane, A., Kreuzer, M., Kuhla, B., Lessire, F., Lund, P., Minnée, E.M.K., Muñoz, C., Niu, M., Nozière, P., Pacheco, D., Prestløkken, E., Reynolds, C.K., Schwarm, A., Spek, J.W., Terranova, M., Vanhatalo, A., Wattiaux, M.A., Weisbjerg, M.R., Yáñez-Ruiz, D.R., Yu, Z., Kebreab, E., 2022. Prediction of nitrogen excretion from data on dairy cows fed a wide range of diets compiled in an intercontinental database: A meta-analysis. *Journal of Dairy Science* 105, 7462-7481. doi: <https://doi.org/10.3168/jds.2021-20885>.

- Bougouin, A., Leytem, A., Dijkstra, J., Dungan, R.S., Kebreab, E., 2016. Nutritional and environmental effects on ammonia emissions from dairy cattle housing: A meta-analysis. *Journal of Environmental Quality* 45, 1123-1132. doi: <https://doi.org/10.2134/jeq2015.07.0389>.
- Boulton, A., Rushton, J., and Wathes, D., 2015a. The management and associated costs of rearing heifers on UK dairy farms from weaning to conception. *Open Journal of Animal Sciences* 5, 294-308. doi: <https://doi.org/10.4236/ojas.2015.53034>.
- Boulton, A.C., Rushton, J., Wathes, D.C., 2017. An empirical analysis of the cost of rearing dairy heifers from birth to first calving and the time taken to repay these costs. *Animal* 11, 1372-1380. doi: <https://doi.org/10.1017/S1751731117000064>.
- Boulton, A.C.R., Jonathan; Wathes; Claire D., 2015b. A study of dairy heifer rearing practices from birth to weaning and their associated costs on uk dairy farms. *Open Journal of Animal Sciences* 5, 185-197. doi: <https://doi.org/10.4236/ojas.2015.52021>.
- Brickell, J.S., Wathes, D.C., 2011. A descriptive study of the survival of holstein-friesian heifers through to third calving on english dairy farms. *Journal of Dairy Science* 94, 1831-1838. doi: <https://doi.org/10.3168/jds.2010-3710>.
- Brunekreef, B., Harrison, R.M., Künzli, N., Querol, X., Sutton, M.A., Heederik, D.J.J., Sigsgaard, T., 2015. Reducing the health effect of particles from agriculture. *The Lancet Respiratory Medicine* 3, 831-832. doi: [https://doi.org/10.1016/S2213-2600\(15\)00413-0](https://doi.org/10.1016/S2213-2600(15)00413-0).

- Bugueiro, A., Fouz, R., Camino, F., Yus, E., Diéguez, F.J., 2019. Robot milking and relationship with culling rate in dairy cows. *Animal* 13, 1304-1310. doi: <https://doi.org/10.1017/S1751731118002896>.
- Burgers, E.E.A., Kok, A., Goselink, R.M.A., Hogeveen, H., Kemp, B., van Knegsel, A.T.M., 2021. Effects of extended voluntary waiting period from calving until first insemination on body condition, milk yield, and lactation persistency. *Journal of Dairy Science* 104, 8009-8022. doi: <https://doi.org/10.3168/jds.2020-19914>.
- Bussink, D.W., Oenema, O., 1998. Ammonia volatilization from dairy farming systems in temperate areas: A review. *Nutrient Cycling in Agroecosystems* 51, 19-33. doi: <https://doi.org/10.1023/A:1009747109538>.
- Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., van Vuuren, A.M., 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184-1196. doi: <https://doi.org/10.1017/S1751731110000911>.
- Cambra-López, M., Aarnink, A.J., Zhao, Y., Calvet, S., Torres, A.G., 2010. Airborne particulate matter from livestock production systems: A review of an air pollution problem. *Environmental Pollution* 158, 1-17. doi: <https://doi.org/10.1016/j.envpol.2009.07.011>.
- CDIC, 2024. Canadian dairy information centre. Retrieved on 6th August 2024 from <https://agriculture.canada.ca/en/sector/animal-industry/canadian-dairy-information-centre/statistics-market-information/dairy-animal-genetics/culling-replacement>.
- Chapman, C.E., Hill, T.M., Elder, D.R., Erickson, P.S., 2017. Nitrogen utilization, preweaning nutrient digestibility, and growth effects of

- holstein dairy calves fed 2 amounts of a moderately high protein or conventional milk replacer. *Journal of Dairy Science* 100, 279-292. doi: <https://doi.org/10.3168/jds.2016-11886>.
- Chiumia, D., Chagunda, M.G., Macrae, A.I., Roberts, D.J., 2013. Predisposing factors for involuntary culling in holstein-friesian dairy cows. *Journal of Dairy Research* 80, 45-50. doi: <https://doi.org/10.1017/s002202991200060x>.
- Clasen, J.B., Kargo, M., Østergaard, S., Fikse, W.F., Rydhmer, L., Strandberg, E., 2021. Genetic consequences of terminal crossbreeding, genomic test, sexed semen, and beef semen in dairy herds. *Journal of Dairy Science* 104, 8062-8075. doi: <https://doi.org/10.3168/jds.2020-20028>.
- Coblentz, W.K., Esser, N.M., Hoffman, P.C., Akins, M.S., 2015. Growth performance and sorting characteristics of corn silage-alfalfa haylage diets with or without forage dilution offered to replacement holstein dairy heifers. *Journal of Dairy Science* 98, 8018-8034. doi: <https://doi.org/10.3168/jds.2015-9491>.
- Coffey, M.P., Hickey, J., Brotherstone, S., 2006. Genetic aspects of growth of holstein-friesian dairy cows from birth to maturity. *Journal of Dairy Science* 89, 322-329. doi: [https://doi.org/10.3168/jds.S0022-0302\(06\)72097-5](https://doi.org/10.3168/jds.S0022-0302(06)72097-5).
- Compton, C.W.R., Heuer, C., Thomsen, P.T., Carpenter, T.E., Phyn, C.V.C., McDougall, S., 2017. Invited review: A systematic literature review and meta-analysis of mortality and culling in dairy cattle. *Journal of Dairy Science* 100, 1-16. doi: <https://doi.org/10.3168/jds.2016-11302>.

- Cooke, J., Cheng, Z., Bourne, N., Wathes, D., 2013. Association between growth rates, age at first calving and subsequent fertility, milk production and survival in holstein-friesian heifers. *Open Journal of Animal Sciences* 3, 1-12. doi: <https://doi.org/10.4236/ojas.2013.31001>.
- Cue, R.I., Pietersma, D., Lefebvre, D., Lacroix, R., Wade, K., Pellerin, D., Passillé, A.-M.d., Rushen, J., 2012. Growth modeling of dairy heifers in québec based on random regression. *Canadian Journal of Animal Science* 92, 33-47. doi: <https://doi.org/10.4141/cjas2011-083>.
- Dallago, G.M., Wade, K.M., Cue, R.I., McClure, J.T., Lacroix, R., Pellerin, D., Vasseur, E., 2021. Keeping dairy cows for longer: A critical literature review on dairy cow longevity in high milk-producing countries. *Animals* 11 doi: <https://doi.org/10.3390/ani11030808>.
- Davidson, S., Hopkins, B.A., Diaz, D.E., Bolt, S.M., Brownie, C., Fellner, V., Whitlow, L.W., 2003. Effects of amounts and degradability of dietary protein on lactation, nitrogen utilization, and excretion in early lactation holstein cows. *Journal of Dairy Science* 86, 1681-1689. doi: [https://doi.org/10.3168/jds.S0022-0302\(03\)73754-0](https://doi.org/10.3168/jds.S0022-0302(03)73754-0).
- Davis Rincker, L.E., VandeHaar, M.J., Wolf, C.A., Liesman, J.S., Chapin, L.T., Weber Nielsen, M.S., 2011. Effect of intensified feeding of heifer calves on growth, pubertal age, calving age, milk yield, and economics. *Journal of Dairy Science* 94, 3554-3567. doi: <https://doi.org/10.3168/jds.2010-3923>.
- De Vries, A., 2017. Economic trade-offs between genetic improvement and longevity in dairy cattle. *Journal of Dairy Science* 100, 4184-4192. doi: <https://doi.org/10.3168/jds.2016-11847>.

- De Vries, A., Marcondes, M.I., 2020. Review: Overview of factors affecting productive lifespan of dairy cows. *Animal* 14, s155-s164. doi: <https://doi.org/10.1017/s1751731119003264>.
- Dhakal, S., J.C. Minx, F.L. Toth, A. Abdel-Aziz, M.J. Figueroa Meza, K. Hubacek, I.G.C. Jonckheere, Yong-Gun Kim, G.F. Nemet, S. Pachauri, X.C. Tan, Wiedmann, T., 2022. Emissions trends and drivers. In *Ipcc, 2022: Climate change 2022: Mitigation of climate change. Contribution of working group iii to the sixth assessment report of the intergovernmental panel on climate change* (ed. P.R. Shukla, J. Skea, R. Slade, A. Al Khourdajie, R. van Diemen, D. McCollum, M. Pathak, S. Some, P. Vyas, R. Fradera, M. Belkacemi, A. Hasija, G. Lisboa, S. Luz and Malley, J.), Cambridge University Press, Cambridge, UK and New York, NY, USA, doi: <https://doi.org/10.1017/9781009157926.004>.
- Dijkstra, J., Bannink, A., Bosma, P.M., Lantinga, E.A., Reijs, J.W., 2018. Modeling the effect of nutritional strategies for dairy cows on the composition of excreta nitrogen. *Frontiers in Sustainable Food Systems* 2 doi: <https://doi.org/10.3389/fsufs.2018.00063>.
- Dijkstra, J., Oenema, O., van Groenigen, J.W., Spek, J.W., van Vuuren, A.M., Bannink, A., 2013. Diet effects on urine composition of cattle and n₂o emissions. *Animal* 7, 292-302. doi: <https://doi.org/10.1017/S1751731113000578>.
- Dingwell, R.T., Wallace, M.M., McLaren, C.J., Leslie, C.F., Leslie, K.E., 2006. An evaluation of two indirect methods of estimating body weight in holstein calves and heifers. *Journal of Dairy Science* 89, 3992-3998. doi: [https://doi.org/10.3168/jds.S0022-0302\(06\)72442-0](https://doi.org/10.3168/jds.S0022-0302(06)72442-0).

- Eastham, N.T., Coates, A., Cripps, P., Richardson, H., Smith, R., Oikonomou, G., 2018. Associations between age at first calving and subsequent lactation performance in uk holstein and holstein-friesian dairy cows. *Plos One* 13, e0197764. doi: <https://doi.org/10.1371/journal.pone.0197764>.
- EC, 2022. E.U. Agricultural outlook for markets, income and environment, 2022-2032. European Commission, DG Agriculture and Rural Development, Brussels.
- Eckert, E., Brown, H.E., Leslie, K.E., DeVries, T.J., Steele, M.A., 2015. Weaning age affects growth, feed intake, gastrointestinal development, and behavior in holstein calves fed an elevated plane of nutrition during the preweaning stage. *Journal of Dairy Science* 98, 6315-6326. doi: <https://doi.org/10.3168/jds.2014-9062>.
- EEA, 2022. European union emission inventory report 1990-2021 under the unece convention on long-range transboundary air pollution (air convention). 9789294805904. European Environment Agency.
- EPA, 2024. Inventory of U.S. Greenhouse gas emissions and sinks: 1990-2022. EPA 430-R-24-004. U.S. Environmental Protection Agency.
- Erickson, P.S., Anderson, J.L., Kalscheur, K.F., Lascano, G.J., Akins, M.S., Heinrichs, A.J., 2020. Symposium review: Strategies to improve the efficiency and profitability of heifer raising. *Journal of Dairy Science* 103, 5700-5708. doi: <https://doi.org/10.3168/jds.2019-17419>.
- Erisman, J.W., Galloway, J.N., Seitzinger, S., Bleeker, A., Dise, N.B., Petrescu, A.M.R., Leach, A.M., de Vries, W., 2013. Consequences of human modification of the global nitrogen cycle. *Philosophical Transactions of*

- the Royal Society B: Biological Sciences 368, 20130116. doi: <https://doi.org/10.1098/rstb.2013.0116>.
- Erisman, J.W., Leach, A., Bleeker, A., Atwell, B., Cattaneo, L., Galloway, J., 2018. An integrated approach to a nitrogen use efficiency (nue) indicator for the food production–consumption chain. Sustainability 10, 925. doi: <https://doi.org/10.3390/su10040925>.
- Ettema, J.F., Santos, J.E.P., 2004. Impact of age at calving on lactation, reproduction, health, and income in first-parity holsteins on commercial farms. Journal of Dairy Science 87, 2730-2742. doi: [https://doi.org/10.3168/jds.S0022-0302\(04\)73400-1](https://doi.org/10.3168/jds.S0022-0302(04)73400-1).
- Faber, S.N., Faber, N.E., McCauley, T.C., Ax, R.L., 2005. Case study: Effects of colostrum ingestion on lactational performance. The Professional Animal Scientist 21, 420-425. doi: [https://doi.org/10.15232/S1080-7446\(15\)31240-7](https://doi.org/10.15232/S1080-7446(15)31240-7).
- Ferrari, V., Galluzzo, F., van Kaam, J.B.C.H.M., Penasa, M., Marusi, M., Finocchiaro, R., Visentin, G., Cassandro, M., 2024. Genetic and genomic evaluation of age at first calving in italian holsteins. Journal of Dairy Science 107, 3104-3113. doi: <https://doi.org/10.3168/jds.2023-23493>.
- Ferrell, C.L., 1982. Effects of postweaning rate of gain on onset of puberty and productive performance of heifers of different breeds. Journal of Animal Science 55, 1272-1283. doi: <https://doi.org/10.2527/jas1982.5561272x>.
- Fetrow, J., Nordlund, K.V., Norman, H.D., 2006. Invited review: Culling: Nomenclature, definitions, and recommendations. Journal of Dairy

- Science 89, 1896-1905. doi: [https://doi.org/10.3168/jds.S0022-0302\(06\)72257-3](https://doi.org/10.3168/jds.S0022-0302(06)72257-3).
- Firkins, J.L., 1996. Maximizing microbial protein synthesis in the rumen. The Journal of Nutrition 126, 1347S-1354S. doi: https://doi.org/10.1093/jn/126.suppl_4.1347S.
- Firkins, J.L., Reynolds, C.K., 2005. Whole-animal nitrogen balance in cattle. In Nitrogen and phosphorus nutrition of cattle: Reducing the environmental impact of cattle operations (ed. Pfeffer, E., Hristov, A.N.), CAB International, Wallingford, UK, pp. 167–186. <https://doi.org/10.1079/9780851990132.0167>.
- Fischer, A.J., Villot, C., van Niekerk, J.K., Yohe, T.T., Renaud, D.L., Steele, M.A., 2019. Invited review: Nutritional regulation of gut function in dairy calves: From colostrum to weaning. Applied Animal Science 35, 498-510. doi: <https://doi.org/10.15232/aas.2019-01887>.
- Fitzhugh, H.A., Jr., 1976. Analysis of growth curves and strategies for altering their shape. Journal of Animal Science 42, 1036-1051. doi: <https://doi.org/10.2527/jas1976.4241036x>.
- Flower, F.C., Weary, D.M., 2003. The effects of early separation on the dairy cow and calf. Animal Welfare 12, 339-348. doi: <https://doi.org/10.1017/S0962728600025847>.
- Foskolos, A., Moorby, J.M., 2018. Evaluating lifetime nitrogen use efficiency of dairy cattle: A modelling approach. Plos One 13, e0201638. doi: <https://doi.org/10.1371/journal.pone.0201638>.
- Fowler, D., Coyle, M., Skiba, U., Sutton, M.A., Cape, J.N., Reis, S., Sheppard, L.J., Jenkins, A., Grizzetti, B., Galloway, J.N., Vitousek, P., Leach, A.,

- Bouwman, A.F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., Voss, M., 2013. The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368, 20130164. doi: <https://doi.org/10.1098/rstb.2013.0164>.
- Fox, D.G., Sniffen, C.J., O'Connor, J.D., Russell, J.B., Van Soest, P.J., 1992. A net carbohydrate and protein system for evaluating cattle diets: Iii. Cattle requirements and diet adequacy. *Journal of Anim Science* 70, 3578-3596. doi: <https://doi.org/10.2527/1992.70113578x>.
- Fox, D.G., Van Amburgh, M.E., Tylutki, T.P., 1999. Predicting requirements for growth, maturity, and body reserves in dairy cattle. *Journal of Dairy Science* 82, 1968-1977. doi: [https://doi.org/10.3168/jds.S0022-0302\(99\)75433-0](https://doi.org/10.3168/jds.S0022-0302(99)75433-0).
- Frank, B., Swensson, C., 2002. Relationship between content of crude protein in rations for dairy cows and milk yield, concentration of urea in milk and ammonia emissions. *Journal of Dairy Science* 85, 1829-1838. doi: [https://doi.org/10.3168/jds.S0022-0302\(02\)74257-4](https://doi.org/10.3168/jds.S0022-0302(02)74257-4).
- Funston, R.N., Martin, J.L., Larson, D.M., Roberts, A.J., 2012. Physiology and endocrinology symposium: Nutritional aspects of developing replacement heifers. *Journal of Animal Science* 90, 1166-1171. doi: <https://doi.org/10.2527/jas.2011-4569>.
- Funston, R.N., Summers, A.F., 2013. Effect of prenatal programming on heifer development. *Veterinary Clinics: Food Animal Practice* 29, 517-536. doi: <https://doi.org/10.1016/j.cvfa.2013.07.001>.

- Gabler, M.T., Heinrichs, A.J., 2003. Effects of increasing dietary protein on nutrient utilization in heifers. *Journal of Dairy Science* 86, 2170-2177. doi: [https://doi.org/10.3168/jds.S0022-0302\(03\)73807-7](https://doi.org/10.3168/jds.S0022-0302(03)73807-7).
- Gabler, M.T., Tozer, P.R., Heinrichs, A.J., 2000. Development of a cost analysis spreadsheet for calculating the costs to raise a replacement dairy heifer. *Journal of Dairy Science* 83, 1104-1109. doi: [https://doi.org/10.3168/jds.S0022-0302\(00\)74975-7](https://doi.org/10.3168/jds.S0022-0302(00)74975-7).
- Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., Cosby, B.J., 2003. The nitrogen cascade. *BioScience* 53, 341-356. doi: [https://doi.org/10.1641/0006-3568\(2003\)053\[0341:Tnc\]2.0.Co;2](https://doi.org/10.1641/0006-3568(2003)053[0341:Tnc]2.0.Co;2).
- Garcia, M., Greco, L.F., Block, E., Santos, J.E.P., Thatcher, W.W., Staples, C.R., 2016. Programming effect of dietary fatty acids on performance of holstein heifers from birth through first lactation. *Animal Feed Science and Technology* 222, 64-74. doi: <https://doi.org/10.1016/j.anifeedsci.2016.10.003>.
- Gonzalez-Mejia, A., Styles, D., Wilson, P., Gibbons, J., 2018. Metrics and methods for characterizing dairy farm intensification using farm survey data. *Plos One* 13, e0195286. doi: <https://doi.org/10.1371/journal.pone.0195286>.
- Green, T.C., Jago, J.G., Macdonald, K.A., Waghorn, G.C., 2013. Relationships between residual feed intake, average daily gain, and feeding behavior in growing dairy heifers. *Journal of Dairy Science* 96, 3098-3107. doi: <https://doi.org/10.3168/jds.2012-6087>.

- Grosshans, T., Xu, Z.Z., Burton, L.J., Johnson, D.L., Macmillan, K.L., 1997. Performance and genetic parameters for fertility of seasonal dairy cows in new zealand. *Livestock Production Science* 51, 41-51. doi: [https://doi.org/10.1016/S0301-6226\(97\)00104-8](https://doi.org/10.1016/S0301-6226(97)00104-8).
- Grummer, R.R., Mashek, D.G., Hayirli, A., 2004. Dry matter intake and energy balance in the transition period. *Veterinary Clinics of North America: Food Animal Practice* 20, 447-470. doi: <https://doi.org/10.1016/j.cvfa.2004.06.013>.
- Habel, J., Uhlig, V., Hoischen-Taubner, S., Schwabenbauer, E.-M., Rumphorst, T., Ebert, L., Möller, D., Sundrum, A., 2021. Income over service life cost-estimation of individual profitability of dairy cows at time of death reveals farm-specific economic trade-offs. *Livestock Science* 254, 104765. doi: <https://doi.org/10.1016/j.livsci.2021.104765>.
- Hadley, G.L., Wolf, C.A., Harsh, S.B., 2006. Dairy cattle culling patterns, explanations, and implications. *Journal of Dairy Science* 89, 2286-2296. doi: [https://doi.org/10.3168/jds.S0022-0302\(06\)72300-1](https://doi.org/10.3168/jds.S0022-0302(06)72300-1).
- Han, L., Heinrichs, A.J., De Vries, A., Dechow, C.D., 2021. Relationship of body weight at first calving with milk yield and herd life. *Journal of Dairy Science* 104, 397-404. doi: <https://doi.org/10.3168/jds.2020-19214>.
- Handcock, R.C., Jenkinson, C.M.C., Laven, R., McNaughton, L.R., Lopez-Villalobos, N., Back, P.J., Hickson, R.E., 2021. Linear versus seasonal growth of dairy heifers decreased age at puberty but did not affect first lactation milk production. *New Zealand Journal of Agricultural Research* 64, 83-100. doi: <https://doi.org/10.1080/00288233.2019.1607404>.

- Handcock, R.C., Lopez-Villalobos, N., McNaughton, L.R., Back, P.J., Edwards, G.R., Hickson, R.E., 2019a. Live weight and growth of holstein-friesian, jersey and crossbred dairy heifers in new zealand. *New Zealand Journal of Agricultural Research* 62, 173-183. doi: <https://doi.org/10.1080/00288233.2018.1465984>.
- Handcock, R.C., Lopez-Villalobos, N., McNaughton, L.R., Back, P.J., Edwards, G.R., Hickson, R.E., 2019b. Positive relationships between body weight of dairy heifers and their first-lactation and accumulated three-parity lactation production. *Journal of Dairy Science* 102, 4577-4589. doi: <https://doi.org/10.3168/jds.2018-15229>.
- Handcock, R.C., Lopez-Villalobos, N., McNaughton, L.R., Back, P.J., Edwards, G.R., Hickson, R.E., 2020. Body weight of dairy heifers is positively associated with reproduction and stayability. *Journal of Dairy Science* 103, 4466-4474. doi: <https://doi.org/10.3168/jds.2019-17545>.
- Heinrichs, A.J., 1993. Raising dairy replacements to meet the needs of the 21st century. *Journal of Dairy Science* 76, 3179-3187. doi: [https://doi.org/10.3168/jds.S0022-0302\(93\)77656-0](https://doi.org/10.3168/jds.S0022-0302(93)77656-0).
- Heinrichs, A.J., Erb, H.N., Rogers, G.W., Cooper, J.B., Jones, C.M., 2007. Variability in holstein heifer heart-girth measurements and comparison of prediction equations for live weight. *Preventive Veterinary Medicine* 78, 333-338. doi: <https://doi.org/10.1016/j.prevetmed.2006.11.002>.
- Heinrichs, A.J., Jones, C.M., Gray, S.M., Heinrichs, P.A., Cornelisse, S.A., Goodling, R.C., 2013. Identifying efficient dairy heifer producers using production costs and data envelopment analysis. *Journal of Dairy Science* 96, 7355-7362. doi: <https://doi.org/10.3168/jds.2012-6488>.

- Heinrichs, A.J., Rogers, G.W., Cooper, J.B., 1992. Predicting body weight and wither height in holstein heifers using body measurements. *Journal of Dairy Science* 75, 3576-3581. doi: [https://doi.org/10.3168/jds.S0022-0302\(92\)78134-X](https://doi.org/10.3168/jds.S0022-0302(92)78134-X).
- Heinrichs, A.J., Zanton, G.I., Lascano, G.J., Jones, C.M., 2017. A 100-year review: A century of dairy heifer research. *Journal of Dairy Science* 100, 10173-10188. doi: <https://doi.org/10.3168/jds.2017-12998>.
- Higgs, R.J., Chase, L.E., Ross, D.A., Van Amburgh, M.E., 2015. Updating the cornell net carbohydrate and protein system feed library and analyzing model sensitivity to feed inputs. *Journal of Dairy Science* 98, 6340-6360. doi: <https://doi.org/10.3168/jds.2015-9379>.
- Hochberg, Z., Feil, R., Constancia, M., Fraga, M., Junien, C., Carel, J.C., Boileau, P., Le Bouc, Y., Deal, C.L., Lillycrop, K., Scharfmann, R., Sheppard, A., Skinner, M., Szyf, M., Waterland, R.A., Waxman, D.J., Whitelaw, E., Ong, K., Albertsson-Wikland, K., 2011. Child health, developmental plasticity, and epigenetic programming. *Endocrine Reviews* 32, 159-224. doi: <https://doi.org/10.1210/er.2009-0039>.
- Hoffman, P.C., 1997. Optimum body size of holstein replacement heifers. *Journal of Animal Science* 75, 836-845. doi: <https://doi.org/10.2527/1997.753836x>.
- Hoffman, P.C., 2017. Feeding management of the dairy heifer from 4 months to calving. In *Large dairy herd management* (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA, p. 431-443. doi: <https://doi.org/10.3168/ldhm.0632>.

- Hoffman, P.C., Esser, N.M., Bauman, L.M., Denzine, S.L., Engstrom, M., Chester-Jones, H., 2001. Short communication: Effect of dietary protein on growth and nitrogen balance of holstein heifers. *Journal of Dairy Science* 84, 843-847. doi: [https://doi.org/10.3168/jds.S0022-0302\(01\)74542-0](https://doi.org/10.3168/jds.S0022-0302(01)74542-0).
- Hoffman, P.C., Simson, C.R., Wattiaux, M., 2007. Limit feeding of gravid holstein heifers: Effect on growth, manure nutrient excretion, and subsequent early lactation performance. *Journal of Dairy Science* 90, 946-954. doi: [https://doi.org/10.3168/jds.S0022-0302\(07\)71578-3](https://doi.org/10.3168/jds.S0022-0302(07)71578-3).
- Hoffman, P.C., Weigel, K.A., Wernberg, R.M., 2008. Evaluation of equations to predict dry matter intake of dairy heifers. *Journal of Dairy Science* 91, 3699-3709. doi: <https://doi.org/10.3168/jds.2007-0644>.
- Hoover, W.H., Stokes, S.R., 1991. Balancing carbohydrates and proteins for optimum rumen microbial yield¹. *Journal of Dairy Science* 74, 3630-3644. doi: [https://doi.org/10.3168/jds.S0022-0302\(91\)78553-6](https://doi.org/10.3168/jds.S0022-0302(91)78553-6).
- Hristov, A.N., Hanigan, M., Cole, A., Todd, R., McAllister, T.A., Ndegwa, P.M., Rotz, A., 2011. Review: Ammonia emissions from dairy farms and beef feedlots. *Canadian Journal of Animal Science* 91, 1-35. doi: <https://doi.org/10.4141/cjas10034>.
- Huhtanen, P., Ahvenjärvi, S., 2022. Review: Problems in determining metabolisable protein value of dairy cow diets and the impact on protein feeding. *Animal* 16, 100539. doi: <https://doi.org/10.1016/j.animal.2022.100539>.
- Huhtanen, P., Hristov, A.N., 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk n

- efficiency in dairy cows. *Journal of Dairy Science* 92, 3222-3232. doi: <https://doi.org/10.3168/jds.2008-1352>.
- Husnain, A., Santos, J.E.P., 2019. Meta-analysis of the effects of prepartum dietary protein on performance of dairy cows. *Journal of Dairy Science* 102, 9791-9813. doi: <https://doi.org/10.3168/jds.2018-16043>.
- Hutchison, J.L., VanRaden, P.M., Null, D.J., Cole, J.B., Bickhart, D.M., 2017. Genomic evaluation of age at first calving. *Journal of Dairy Science* 100, 6853-6861. doi: <https://doi.org/10.3168/jds.2016-12060>.
- IPCC, 1996. Revised 1996 IPCC guidelines for national greenhouse gas inventories. IPCC, Geneva, Switzerland.
- Jasper, J., Weary, D.M., 2002. Effects of ad libitum milk intake on dairy calves. *Journal of Dairy Science* 85, 3054-3058. doi: [https://doi.org/10.3168/jds.S0022-0302\(02\)74391-9](https://doi.org/10.3168/jds.S0022-0302(02)74391-9).
- James, T., Freitas, N., Ashbaugh, L., Meyer, D. 1997. Field estimates of ammonia volatilization from cattle production facilities. In *Emission inventory: planning for the future*, p. 259-267. Proceedings of a Specialty Conference, Research Triangle, NC. 1997 Oct. 28-30. AWMA, Pittsburgh, PA.
- Jensen, L.S., Schjoerring, J.K., van der Hoek, K.W., Poulsen, H.D., Zevenbergen, J.F., Pallière, C., Lammel, J., Brentrup, F., Jongbloed, A.W., Willems, J., van Grinsven, H., 2011. Benefits of nitrogen for food, fibre and industrial production. In *The european nitrogen assessment: Sources, effects and policy perspectives* (ed. Sutton, M.A., Howard, C.M., Erisman, J.W., Billen, G., Bleeker, A., Grennfelt, P., van Grinsven,

- H. and Grizzetti, B.), Cambridge University Press, Cambridge, p. 32-61.
doi: <https://doi.org/10.1017/CBO9780511976988.006>.
- Johansen, M., Madsen, P.A., Brask-Pedersen, D.N., Lund, P., 2022. Effect of age and dietary crude protein level on nitrogen excretion in dairy heifers. *Livestock Science* 264, 105058.
doi: <https://doi.org/10.1016/j.livsci.2022.105058>.
- Jones, G.A., Kammel, D.W., 2017. Large dairy herd design and systems in temperate and cold climates. In *Large dairy herd management* (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA, p. 71-82. doi: <https://doi.org/10.3168/lidhm.0632>.
- Kelly, P., Shalloo, L., Wallace, M., Dillon, P., 2020. The Irish dairy industry – recent history and strategy, current state and future challenges. *International Journal of Dairy Technology* 73, 309-323. doi: <https://doi.org/10.1111/1471-0307.12682>.
- Kertz, A.F., Hill, T.M., Quigley, J.D., Heinrichs, A.J., Linn, J.G., Drackley, J.K., 2017. A 100-year review: Calf nutrition and management. *Journal of Dairy Science* 100, 10151-10172. doi: <https://doi.org/10.3168/jds.2017-13062>.
- Key, C., Maciver, R.M., 1985. The effects of maternal influences on sheep: Breed differences in grazing, resting and courtship behaviour. In *Reproductive and developmental behaviour in sheep* (ed. Fraser, A.F.), Elsevier, Amsterdam, p. 415-430. doi: <https://doi.org/10.1016/B978-0-444-42444-0.50043-7>.

- Khan, M.A., Bach, A., Weary, D.M., von Keyserlingk, M.A.G., 2016. Invited review: Transitioning from milk to solid feed in dairy heifers. *Journal of Dairy Science* 99, 885-902. doi: <https://doi.org/10.3168/jds.2015-9975>.
- Khan, M.A., Lee, H.J., Lee, W.S., Kim, H.S., Ki, K.S., Hur, T.Y., Suh, G.H., Kang, S.J., Choi, Y.J., 2007. Structural growth, rumen development, and metabolic and immune responses of holstein male calves fed milk through step-down and conventional methods. *Journal of Dairy Science* 90, 3376-3387. doi: <https://doi.org/10.3168/jds.2007-0104>.
- Khan, M.A., Weary, D.M., Veira, D.M., von Keyserlingk, M.A.G., 2012. Postweaning performance of heifers fed starter with and without hay during the milk-feeding period. *Journal of Dairy Science* 95, 3970-3976. doi: <https://doi.org/10.3168/jds.2011-5027>.
- Khan, M.A., Weary, D.M., von Keyserlingk, M.A., 2011a. Hay intake improves performance and rumen development of calves fed higher quantities of milk. *Journal of Dairy Science* 94, 3547-3553. doi: <https://doi.org/10.3168/jds.2010-3871>.
- Khan, M.A., Weary, D.M., von Keyserlingk, M.A.G., 2011b. Invited review: Effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. *Journal of Dairy Science* 94, 1071-1081. doi: <https://doi.org/10.3168/jds.2010-3733>.
- Kim, K.-H., Kabir, E., Kabir, S., 2015. A review on the human health impact of airborne particulate matter. *Environment International* 74, 136-143. doi: <https://doi.org/10.1016/j.envint.2014.10.005>.
- Kosiorowska, A., Puggaard, L., Hedemann, M.S., Sehested, J., Jensen, S.K., Kristensen, N.B., Kuropka, P., Marycz, K., Vestergaard, M., 2011.

- Gastrointestinal development of dairy calves fed low- or high-starch concentrate at two milk allowances. *Animal* 5, 211-219. doi: <https://doi.org/10.1017/S1751731110001710>.
- Krpalkova, L., Cabrera, E.V., Kvapilik, J., Burdych, J., 2016. Dairy farm profit according to the herd size, milk yield, and number of cows per worker. *Agricultural Economics* 62, 225-234. doi: <https://doi.org/10.17221/126/2015-AGRICECON>.
- Krpálková, L., Cabrera, V.E., Vacek, M., Štípková, M., Stádník, L., Crump, P., 2014. Effect of prepubertal and postpubertal growth and age at first calving on production and reproduction traits during the first 3 lactations in holstein dairy cattle. *Journal of Dairy Science* 97, 3017-3027. doi: <https://doi.org/10.3168/jds.2013-7419>.
- Kruse, K.A., Combs, D.K., Esser, N.M., Coblenz, W.K., Hoffman, P.C., 2010. Evaluation of potential carryover effects associated with limit feeding of gravid holstein heifers. *Journal of Dairy Science* 93, 5374-5384. doi: <https://doi.org/10.3168/jds.2010-3401>.
- Kulkarni, P.S., Mourits, M.C.M., Slob, J., Veldhuis, A.M.B., Nielen, M., Hogeveen, H., Schaik, G.v., Steeneveld, W., 2023. Dutch dairy farmers' perspectives on culling reasons and strategies. *Preventive Veterinary Medicine* 218, 105997. doi: <https://doi.org/10.1016/j.prevetmed.2023.105997>.
- Laarman, A.H., Ruiz-Sanchez, A.L., Sugino, T., Guan, L.L., Oba, M., 2012. Effects of feeding a calf starter on molecular adaptations in the ruminal epithelium and liver of holstein dairy calves. *Journal of Dairy Science* 95, 2585-2594. doi: <https://doi.org/10.3168/jds.2011-4788>.

- Lacasse, P., Vanacker, N., Ollier, S., Ster, C., 2018. Innovative dairy cow management to improve resistance to metabolic and infectious diseases during the transition period. *Research in Veterinary Science* 116, 40-46. doi: <https://doi.org/10.1016/j.rvsc.2017.06.020>.
- Lapierre, H., Larsen, M., Sauvant, D., Van Amburgh, M.E., Van Duinkerken, G., 2018. Review: Converting nutritional knowledge into feeding practices: A case study comparing different protein feeding systems for dairy cows. *Animal* 12, s457-s466. doi: <https://doi.org/10.1017/S1751731118001763>.
- Lascano, G.J., Koch, L.E., Heinrichs, A.J., 2016. Precision-feeding dairy heifers a high rumen-degradable protein diet with different proportions of dietary fiber and forage-to-concentrate ratios. *Journal of Dairy Science* 99, 7175-7190. doi: <https://doi.org/10.3168/jds.2016-11190>.
- Lauber, M.R., Fricke, P.M., 2023. The association between insemination eligibility and reproductive performance of nulliparous heifers on subsequent body weight and milk production of primiparous holstein cows. *JDS Communications* 4, 428-432. doi: <https://doi.org/10.3168/jdsc.2023-0372>.
- Le Cozler, Y., Lollivier, V., Lacasse, P., Disenhaus, C., 2008. Rearing strategy and optimizing first-calving targets in dairy heifers: A review. *Animal* 2, 1393-1404. doi: <https://doi.org/10.1017/S1751731108002498>.
- Lobley, G.E., 1993. Species comparisons of tissue protein metabolism: Effects of age and hormonal action. *The Journal of Nutrition* 123, 337-343. doi: https://doi.org/10.1093/jn/123.suppl_2.337.

- MacPherson, J.A.R., Berends, H., Leal, L.N., Cant, J.P., Martín-Tereso, J., Steele, M.A., 2016. Effect of plane of milk replacer intake and age on glucose and insulin kinetics and abomasal emptying in female holstein friesian dairy calves fed twice daily. *Journal of Dairy Science* 99, 8007-8017. doi: <https://doi.org/10.3168/jds.2015-10826>.
- Maher, P., Good, M., More, S.J., 2008. Trends in cow numbers and culling rate in the irish cattle population, 2003 to 2006. *Irish Veterinary Journal* 61, 455. doi: <https://doi.org/10.1186/2046-0481-61-7-455>.
- Malherbe, L., German, R., Couvidat, F., , Zanatta, L., Blannin, L., James, A., Létinois, L., Schucht, S., Berthelot, B. and Raoult, J., 2022. Emissions of ammonia and methane from the agricultural sector. Emissions from livestock farming (ETC Report 2022/21). European Topic Centre on Human Health and the Environment.
- Marini, J.C., Van Amburgh, M.E., 2003. Nitrogen metabolism and recycling in holstein heifers. *Journal of Animal Science* 81, 545-552. doi: <https://doi.org/10.2527/2003.812545x>.
- Merchen, N.R., Titgemeyer, E.C., 1992. Manipulation of amino acid supply to the growing ruminant. *Journal of Animal Science* 70, 3238-3247. doi: <https://doi.org/10.2527/1992.70103238x>.
- Mirzaei, M., Dadkhah, N., Baghbanzadeh-Nobari, B., Agha-Tehrani, A., Eshraghi, M., Imani, M., Shiasi-Sardoabi, R., Ghaffari, M.H., 2018. Effects of preweaning total plane of milk intake and weaning age on intake, growth performance, and blood metabolites of dairy calves. *Journal of Dairy Science* 101, 4212-4220. doi: <https://doi.org/10.3168/jds.2017-13766>.

- Mitchell, L.K., Heinrichs, A.J., 2020. Increasing grass hay inclusion level on weaned dairy calf growth, intake, digestibility, and ruminal fermentation. *Journal of Dairy Science* 103, 9012-9023. doi: <https://doi.org/10.3168/jds.2020-18596>.
- Mohd nor, N., Steeneveld, W., Mourits, M.C.M., Hogeveen, H., 2012. Estimating the costs of rearing young dairy cattle in the netherlands using a simulation model that accounts for uncertainty related to diseases. *Preventive Veterinary Medicine* 106, 214-224. doi: <https://doi.org/10.1016/j.prevetmed.2012.03.004>.
- Mohd Nor, N., Steeneveld, W., van Werven, T., Mourits, M.C.M., Hogeveen, H., 2013. First-calving age and first-lactation milk production on dutch dairy farms. *Journal of Dairy Science* 96, 981-992. doi: <https://doi.org/10.3168/jds.2012-5741>.
- Molano, R.A., Saito, A., Luchini, D.N., Van Amburgh, M.E., 2020. Effects of rumen-protected methionine or methionine analogs in starter on plasma metabolites, growth, and efficiency of holstein calves from 14 to 91 d of age. *Journal of Dairy Science* 103, 10136-10151. doi: <https://doi.org/10.3168/jds.2020-18630>.
- Monteiro, A.P.A., Tao, S., Thompson, I.M.T., Dahl, G.E., 2016. In utero heat stress decreases calf survival and performance through the first lactation. *Journal of Dairy Science* 99, 8443-8450. doi: <https://doi.org/10.3168/jds.2016-11072>.
- Moore, D.A., Taylor, J., Hartman, M.L., Sischo, W.M., 2009. Quality assessments of waste milk at a calf ranch. *Journal of Dairy Science* 92, 3503-3509. doi: <https://doi.org/10.3168/jds.2008-1623>.

- Nabuurs, G.-J., Mrabet, R., Abu Hatab, A., Bustamante, M., Clark, H., Havlík, P., House, J., Mbow, C., Ninan, K.N., Popp, A., Roe, S., Sohngen, B., Towprayoon, S., 2023. Agriculture, forestry and other land uses (AFOLU). In Climate change 2022 - mitigation of climate change: Working group iii contribution to the sixth assessment report of the intergovernmental panel on climate change (ed. Intergovernmental Panel on Climate, C.), Cambridge University Press, Cambridge, p. 747-860. doi: <https://doi.org/10.1017/9781009157926.009>.
- NASEM, 2021. Nutrient requirements of dairy cattle, 8th revised edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/25806>.
- Neave, H.W., Lomb, J., von Keyserlingk, M.A.G., Behnam-Shabahang, A., Weary, D.M., 2017. Parity differences in the behavior of transition dairy cows. Journal of Dairy Science 100, 548-561. doi: <https://doi.org/10.3168/jds.2016-10987>.
- Norman, H.D., Wright, J.R., Kuhn, M.T., Hubbard, S.M., Cole, J.B., VanRaden, P.M., 2009. Genetic and environmental factors that affect gestation length in dairy cattle. Journal of Dairy Science 92, 2259-2269. doi: <https://doi.org/10.3168/jds.2007-0982>.
- NRC, 1989. Nutrient requirements of dairy cattle. The National Academies Press, Washington, DC.
- NRC, 1996. Nutrient requirements of beef cattle, Seventh Revised Edition. The National Academies press, Washington, DC.

- NRC, 2001. Nutrient requirements of dairy cattle, Seventh Revised Edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/9825>.
- Overton, M., Dhuyvetter, K., 2017. Economic considerations regarding the raising of dairy replacement heifers. In Large dairy herd management (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA, p. 457-474. doi: <https://doi.org/10.3168/ldhm.0632>.
- Overton, M.W., Dhuyvetter, K.C., 2020. Symposium review: An abundance of replacement heifers: What is the economic impact of raising more than are needed? Journal of Dairy Science 103, 3828-3837. doi: <https://doi.org/10.3168/jds.2019-17143>.
- Perotto, D., Cue, R.I., Lee, A.J., 1992. Comparison of nonlinear functions for describing the growth curve of three genotypes of dairy cattle. Canadian Journal of Animal Science 72, 773-782. doi: <https://doi.org/10.4141/cjas92-089>.
- Place, S.E., Mitloehner, F.M., 2010. Invited review: Contemporary environmental issues: A review of the dairy industry's role in climate change and air quality and the potential of mitigation through improved production efficiency. Journal of Dairy Science 93, 3407-3416. doi: <https://doi.org/10.3168/jds.2009-2719>.
- Powell, J.M., Broderick, G.A., Misselbrook, T.H., 2008a. Seasonal diet affects ammonia emissions from tie-stall dairy barns. Journal of Dairy Science 91, 857-869. doi: <https://doi.org/10.3168/jds.2007-0588>.
- Powell, J.M., Misselbrook, T.H., Casler, M.D., 2008b. Season and bedding impacts on ammonia emissions from tie-stall dairy barns. Journal of

- Environmental Quality 37, 7-15. doi:
<https://doi.org/10.2134/jeq2007.0282>.
- Pytlewski, J., Antkowiak, I., Staniek, M., Walendowska, A., Skrzypek, R., 2010. The association between reproductive disorders and lifetime performance in polish holstein-friesian cows. *Acta Scientiarum Polonorum Zootechnica* 9, 51-62.
- Raboisson, D., Cahuzac, E., Sans, P., Allaire, G., 2011. Herd-level and contextual factors influencing dairy cow mortality in france in 2005 and 2006. *Journal of Dairy Science* 94, 1790-1803. doi:
<https://doi.org/10.3168/jds.2010-3634>.
- Rasby, R., 2007. Early weaning beef calves. *Veterinary Clinics of North America: Food Animal Practice* 23, 29-40. doi:
<https://doi.org/10.1016/j.cvfa.2007.01.002>.
- Reinhardt, V., Mutiso, F.M., Reinhardt, A., 1978. Social behaviour and social relationships between female and male prepubertal bovine calves (*bos indicus*). *Applied Animal Ethology* 4, 43-54. doi:
[https://doi.org/10.1016/0304-3762\(78\)90092-5](https://doi.org/10.1016/0304-3762(78)90092-5).
- Rilanto, T., Viidu, D.-A., Kaart, T., Orro, T., Viltrop, A., Emanuelson, U., Ferguson, E., Mõtus, K., 2022. Attitudes and personality of farm managers and association with cow culling rates and longevity in large-scale commercial dairy farms. *Research in Veterinary Science* 142, 31-42. doi: <https://doi.org/10.1016/j.rvsc.2021.11.006>.
- Robles, V., González, L.A., Ferret, A., Manteca, X., Calsamiglia, S., 2007. Effects of feeding frequency on intake, ruminal fermentation, and feeding

- behavior in heifers fed high-concentrate diets. *Journal of Animal Science* 85, 2538-2547. doi: <https://doi.org/10.2527/jas.2006-739>.
- Russell, J.B., O'Connor, J.D., Fox, D.G., Van Soest, P.J., Sniffen, C.J., 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *Journal of Animal Science* 70, 3551-3561. doi: <https://doi.org/10.2527/1992.70113551x>.
- Sander, E.G., Warner, R.G., Harrison, H.N., Loosli, J.K., 1959. The stimulatory effect of sodium butyrate and sodium propionate on the development of rumen mucosa in the young calf. *Journal of Dairy Science* 42, 1600-1605. doi: [https://doi.org/10.3168/jds.S0022-0302\(59\)90772-6](https://doi.org/10.3168/jds.S0022-0302(59)90772-6).
- Schillo, K.K., Hall, J.B., Hileman, S.M., 1992. Effects of nutrition and season on the onset of puberty in the beef heifer. *Journal of Animal Science* 70, 3994-4005. doi: <https://doi.org/10.2527/1992.70123994x>.
- Sguizzato, A.L.L., Marcondes, M.I., Dijkstra, J., Valadares Filho, S.d.C., Campos, M.M., Machado, F.S., Silva, B.C., Rotta, P.P., 2020. Energy requirements for pregnant dairy cows. *Plos One* 15, e0235619. doi: <https://doi.org/10.1371/journal.pone.0235619>.
- Shivley, C.B., Lombard, J.E., Urie, N.J., Kopral, C.A., Santin, M., Earleywine, T.J., Olson, J.D., Garry, F.B., 2018. Preweaned heifer management on us dairy operations: Part vi. Factors associated with average daily gain in preweaned dairy heifer calves. *Journal of Dairy Science* 101, 9245-9258. doi: <https://doi.org/10.3168/jds.2017-14022>.
- Shook, G.E., 2006. Major advances in determining appropriate selection goals. *Journal of Dairy Science* 89, 1349-1361. doi: [https://doi.org/10.3168/jds.S0022-0302\(06\)72202-0](https://doi.org/10.3168/jds.S0022-0302(06)72202-0).

- Siafakas, S., Tsiplakou, E., Kotsarinis, M., Tsiboukas, K., Zervas, G., 2019. Identification of efficient dairy farms in greece based on home grown feedstuffs, using the data envelopment analysis method. *Livestock Science* 222, 14-20. doi: <https://doi.org/10.1016/j.livsci.2019.02.008>.
- Silva, A.L., Detmann, E., Rennó, L.N., Pedroso, A.M., Fontes, M.M.S., Morais, V.C., Sguizzato, A.L.L., Abreu, M.B., Rotta, P.P., Marcondes, M.I., 2018. Effects of rumen undegradable protein on intake, digestibility and rumen kinetics and fermentation characteristics of dairy heifers. *Animal Feed Science and Technology* 244, 1-10. doi: <https://doi.org/10.1016/j.anifeedsci.2018.07.019>.
- Silva, A.L., DeVries, T.J., Fernandes, E.C., Marcondes, M.I., 2021. Short communication: Development and evaluation of equations to predict growth of holstein dairy heifers in a tropical climate. *Journal of Dairy Science* 104, 525-531. doi: <https://doi.org/10.3168/jds.2020-18624>.
- Soberon, F., Raffrenato, E., Everett, R.W., Van Amburgh, M.E., 2012. Preweaning milk replacer intake and effects on long-term productivity of dairy calves. *Journal of Dairy Science* 95, 783-793. doi: <https://doi.org/10.3168/jds.2011-4391>.
- Stěhulová, I., Lidfors, L., Špinka, M., 2008. Response of dairy cows and calves to early separation: Effect of calf age and visual and auditory contact after separation. *Applied Animal Behaviour Science* 110, 144-165. doi: <https://doi.org/10.1016/j.applanim.2007.03.028>.
- Storm, E., Orskov, E.R., 1983. The nutritive value of rumen micro-organisms in ruminants. Large-scale isolation and chemical composition of rumen

- micro-organisms. *British Journal of Nutrition* 50, 463-470. doi: <https://doi.org/10.1079/bjn19830114>.
- Su, H., Akins, M.S., Esser, N.M., Ogden, R., Coblenz, W.K., Kalscheur, K.F., Hatfield, R., 2017. Effects of feeding alfalfa stemlage or wheat straw for dietary energy dilution on nutrient intake and digestibility, growth performance, and feeding behavior of holstein dairy heifers. *Journal of Dairy Science* 100, 7106-7115. doi: <https://doi.org/10.3168/jds.2016-12448>.
- Sutton, M.A., van Grinsven, H., Billen, G., Bleeker, A., Bouwman, A.F., Bull, K., Erisman, J.W., Grennfelt, P., Grizzetti, B., Howard, C.M., Oenema, O., Spranger, T., Winiwarter, W., 2011. Summary for policy makers. In *The European Nitrogen Assessment: Sources, effects and policy perspectives* (ed. Bleeker, A., Grizzetti, B., Howard, C.M., Billen, G., van Grinsven, H., Erisman, J.W., Sutton, M.A. and Grennfelt, P.), Cambridge University Press, Cambridge, UK, p. xxiv-xxxiv. doi: <https://doi.org/10.1017/CBO9780511976988.002>.
- Tamate, H., McGilliard, A.D., Jacobson, N.L., Getty, R., 1962. Effect of various dietaries on the anatomical development of the stomach in the calf¹. *Journal of Dairy Science* 45, 408-420. doi: [https://doi.org/10.3168/jds.S0022-0302\(62\)89406-5](https://doi.org/10.3168/jds.S0022-0302(62)89406-5).
- Tamminga, S., 1996. A review on environmental impacts of nutritional strategies in ruminants. *Journal of Animal Science* 74, 3112-3124. doi: <https://doi.org/10.2527/1996.74123112x>.
- Tedeschi, L.O., Fox, D.G., Fonseca, M.A., Cavalcanti, L.F.L., 2015. Models of protein and amino acid requirements for cattle. *Revista Brasileira de*

Zootecnia 44, 109-132. doi: <https://doi.org/10.1590/S1806-92902015000300005>.

Tomlinson, D.L., James, R.E., Bethard, G.L., McGilliard, M.L., 1997. Influence of undegradability of protein in the diet on intake, daily gain, feed efficiency, and body composition of holstein heifers. Journal of Dairy Science 80, 943-948. doi: [https://doi.org/10.3168/jds.S0022-0302\(97\)76018-1](https://doi.org/10.3168/jds.S0022-0302(97)76018-1).

Tozer, P.R., Heinrichs, A.J., 2001. What affects the costs of raising replacement dairy heifers: A multiple-component analysis. Journal of Dairy Science 84, 1836-1844. doi: [https://doi.org/10.3168/jds.S0022-0302\(01\)74623-1](https://doi.org/10.3168/jds.S0022-0302(01)74623-1).

Tulloch, D., 1979. The water buffalo, bubalus bubalis, in australia: Reproductive and parent-offspring behaviour. Wildlife Research 6, 265-287. doi: <https://doi.org/10.1071/WR9790265>.

Van Amburgh, M.E., Collao-Saenz, E.A., Higgs, R.J., Ross, D.A., Recktenwald, E.B., Raffrenato, E., Chase, L.E., Overton, T.R., Mills, J.K., Foskolos, A., 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. Journal of Dairy Science 98, 6361-6380. doi: <https://doi.org/10.3168/jds.2015-9378>.

Van Amburgh, M.E., Fox, D.G., Galton, D.M., Bauman, D.E., Chase, L.E., 1998a. Evaluation of National Research Council and Cornell Net Carbohydrate and Protein Systems for predicting requirements of holstein heifers. Journal of Dairy Science 81, 509-526. doi: [https://doi.org/10.3168/jds.S0022-0302\(98\)75603-6](https://doi.org/10.3168/jds.S0022-0302(98)75603-6).

- Van Amburgh, M.E., Galton, D.M., Bauman, D.E., Everett, R.W., Fox, D.G., Chase, L.E., Erb, H.N., 1998b. Effects of three prepubertal body growth rates on performance of Holstein heifers during first lactation. *Journal of Dairy Science* 81, 527-538. doi: [https://doi.org/10.3168/jds.S0022-0302\(98\)75604-8](https://doi.org/10.3168/jds.S0022-0302(98)75604-8).
- Van Amburgh, M.E., Soberon, F., Meyer, M.J., Molano, R.A., 2019. Symposium review: Integration of postweaning nutrient requirements and supply with composition of growth and mammary development in modern dairy heifers. *Journal of Dairy Science* 102, 3692-3705. doi: <https://doi.org/10.3168/jds.2018-15270>.
- van Niekerk, J.K., Fischer-Tlustos, A.J., Wilms, J.N., Hare, K.S., Welboren, A.C., Lopez, A.J., Yohe, T.T., Cangiano, L.R., Leal, L.N., Steele, M.A., 2021. ADSA foundation scholar award: New frontiers in calf and heifer nutrition—from conception to puberty. *Journal of Dairy Science* 104, 8341-8362. doi: <https://doi.org/10.3168/jds.2020-20004>.
- Van Soest, P.J., 1994. *Nutritional ecology of the ruminant*. 2nd edition, Cornell University Press, Ithaca, New York, USA.
- Wathes, D.C., Brickell, J.S., Bourne, N.E., Swali, A., Cheng, Z., 2008. Factors influencing heifer survival and fertility on commercial dairy farms. *Animal* 2, 1135-1143. doi: <https://doi.org/10.1017/S1751731108002322>.
- Wathes, D.C., Pollott, G.E., Johnson, K.F., Richardson, H., Cooke, J.S., 2014. Heifer fertility and carry over consequences for life time production in dairy and beef cattle. *Animal* 8, 91-104. doi: <https://doi.org/10.1017/S1751731114000755>.

- Weart, S.R., 2008. The discovery of global warming, Rev. and expanded edition, Harvard University Press, Cambridge, Massachusetts, USA.
- Weary, D.M., Jasper, J., Hötzel, M.J., 2008. Understanding weaning distress. *Applied Animal Behaviour Science* 110, 24-41. doi: <https://doi.org/10.1016/j.applanim.2007.03.025>.
- White, D.S., Duberstein, K.J., Fain Bohlen, J.L., Bertrand, J.K., Nelson, A.H., Froetschel, M.A., Davidson, B.E., Graves, W.M., 2015. Allometric comparison of Georgia dairy heifers on farms and at youth shows. *Journal of Dairy Science* 98, 1345-1353. doi: <https://doi.org/10.3168/jds.2014-8529>.
- Wickersham, T.A., Titgemeyer, E.C., Cochran, R.C., 2009. Methodology for concurrent determination of urea kinetics and the capture of recycled urea nitrogen by ruminal microbes in cattle. *Animal* 3, 372-379. doi: <https://doi.org/10.1017/S1751731108003704>.
- Wood, S., Reyher, K.K., Barrett, D.C., 2015. Comparison of visual assessment and heart girth tape measurement for estimating the weight of cattle in clinical practice. *The Veterinary Journal* 203, 337-338. doi: <https://doi.org/10.1016/j.tvjl.2014.12.034>.
- Wyer, K.E., Kelleghan, D.B., Blanes-Vidal, V., Schauburger, G., Curran, T.P., 2022. Ammonia emissions from agriculture and their contribution to fine particulate matter: A review of implications for human health. *Journal of Environmental Management* 323, 116285. doi: <https://doi.org/10.1016/j.jenvman.2022.116285>.
- Zanton, G.I., Gabler, M.T., Heinrichs, A.J., 2007. Manipulation of soluble and rumen-undegradable protein in diets fed to postpubertal dairy heifers.

Journal of Dairy Science 90, 978-986. doi:
[https://doi.org/10.3168/jds.S0022-0302\(07\)71582-5](https://doi.org/10.3168/jds.S0022-0302(07)71582-5).

Zanton, G.I., Heinrichs, A.J., 2005. Meta-analysis to assess effect of prepubertal average daily gain of Holstein heifers on first-lactation production. Journal of Dairy Science 88, 3860-3867. doi:
[https://doi.org/10.3168/jds.S0022-0302\(05\)73071-X](https://doi.org/10.3168/jds.S0022-0302(05)73071-X).

Zanton, G.I., Heinrichs, A.J., 2016. Efficiency and rumen responses in younger and older holstein heifers limit-fed diets of differing energy density. Journal of Dairy Science 99, 2825-2836. doi:
<https://doi.org/10.3168/jds.2015-10316>.

Chapter 2

Current status of feed nitrogen use efficiency of dairy replacement heifers

2.1. Abstract

Increasing nitrogen use efficiency (NUE) of dairy cattle farms has been proposed as a key solution to mitigate nitrogen pollution. Dairy replacement heifers comprise a considerable portion of animal capital in each herd. Hence, it is important to investigate the factors that impact nitrogen use efficiency (NUE) of replacement heifers and as a consequence farm's overall NUE. The aim of the study was to investigate the management conditions and the current status of NUE in dairy replacement heifers in Greece. Data were collected from 14 dairy farms and information about the overall farm and heifer rearing management were obtained during a 2-day visit. Reproduction records of the herd were retrieved and the age at first service (T_{1stSer}), services/heifer and age at first calving (T_{1stCal}) were determined. Body weight (BW) in different stages was estimated by tape measuring 15-20 % of all heifer groups and the respective stage's average daily gain (ADG) was calculated. Moreover, the mature BW (MBW) of the cows in each herd was estimated, tape measuring 15-20 % of all cows, and used to evaluate growth. Growth targets based on farm's MBW were set as follows: (1) 55% MBW at first service, (2) 85% MBW at calving ($Target_{85}$) and (3) 95% MBW ($Target_{95}$) at calving, and the corresponding ADG targets for each growth stage were set. For ration evaluation, a description of the total mixed ration (TMR) was recorded and feed samples were collected and chemically analyzed. The TMR were evaluated with the Cornell Net Carbohydrate and Protein System (CNCPS v6.5). It was found that T_{1stSer} and T_{1stCal} was 15.4 (± 2.4) and 25.3 months (± 2.65), respectively and pregnancy was achieved with 1.54 services/heifer. Heifer BW at T_{1stSer} and T_{1stCal} was 437.1 kg and 692.8 kg, respectively that was within the

targets of 55 % and 85 % of MBW (404 and 625 for T_{1stSer} and T_{1stCal}, respectively) but they were achieved with a delay of 1 month. In all farms, metabolizable energy was the first limiting factor, while metabolizable protein was supplied above heifers' requirements and was progressively increasing along with heifers age ($P < 0.0001$). Regarding NUE, it was higher from 3 to 12 months, (on average 26.1 %), and was decreasing progressively, from 15 to 24 months (on average 13.0 %). In conclusion, frequent monitoring of BW and different plane of nutrition should be implemented in each growth stage, adjusted to respective requirements to avoid MP oversupply.

2.2. Introduction

In dairy cattle farming, nitrogen (N) is supplied through protein or non-protein forms to serve animal's physiological functions and secure optimum growth and production (Broderick, 2018). However, several practices have been linked with nitrogen pollution, such as the excessive N application through fertilizers, manure storage and application in fields or protein overfeeding of dairy cattle (Galloway et al., 2008; Foskolos and Plomaritou, 2023). This dual role of N challenges dairy farming systems to maintain high productivity, while perform in an environmentally sustainable way. Within the European Union (EU) it has been calculated that each lactating period 3.0 Tg of N are introduced into the ecosystem to feed dairy cattle and 2.2 Tg of N are excreted in feces and urine (Foskolos and Plomaritou, 2023). This is why the EU has dedicated resources to address the issue by developing strategies to mitigate N pollution. Probably the most comprehensive EU funded project on N pollution, the European Nitrogen Assessment, suggested as a key solution to mitigate N pollution the increase of farms' N use efficiency (NUE; Sutton et al., 2011). An

increase in NUE can be achieved either by increasing productive N or by decreasing the feed N supplied to animals (Calsamiglia et al., 2010). However, identifying other factors that impact NUE, will allow a holistic approach to effectively address the issue.

The overall farm NUE is directly affected by replacement heifers, as the later consist a major component of dairy herds (Boulton, 2015; Mohd Nor et al., 2015). Main rearing strategy for Holstein breed is to achieve first calving at 24 months old, as it is regarded the optimal age both from an economical point of view (Ettema and Santos, 2004; Mohd Nor et al., 2013) and in terms of maximizing lifetime milk production (Eastham et al., 2018). However, higher values of age at first calving (AFC) not only extend rearing period of heifers (Overton, 2017), but also affect overall NUE of the dairy herd. Indeed, AFC has been found to impact negatively lifetime NUE of the dairy herd (Foskolos and Moorby, 2018), meaning that higher N losses are occurring with higher age of first calving. Hence, breeding age should be controlled as a first step, to achieve first calving at two years and, therefore, minimize N losses.

Body size and growth rate are main driving forces that define the time of first service (Le Cozler et al., 2008), indicating that except reproduction factors, nutritional management holds an important role in the NUE of the replacement heifers. Indeed, heifer's nutrition has been correlated with the efficiency of N use by replacement heifers, especially, the ratio between metabolizable protein and crude protein (Foskolos and Moorby, 2018). Hence, wider management strategies, related to nutrition, growth and reproduction, are needed to increase NUE of replacement heifers and at the end overall farm NUE. The objective of the current study was to evaluate current feeding strategies and management

conditions of dairy replacement heifers in Greece and define possible areas of improvement.

2.3. Material and methods

2.3.1. Farm visits and questionnaire

The study was conducted in the period between January 2020 and February 2022. Previous work (Siafakas, 2019) on farm typology in Greece, assisted to identify farms with medium and large cluster characteristics. As a next step, 25 farms were approached and the objectives of the visit and farm sampling protocol were explained. Based on their acceptability, 14 dairy farms in total were included. These farms were located in the regions of Thessaly, Macedonia and Thrace. Data collection was accomplished through a two-day visit in each dairy farm to develop a representative overview of the rearing management conditions of the previous rearing period.

Information concerning the overall farm and heifer rearing management was obtained through interview using a questionnaire (Supplementary material). The questionnaire comprised four main areas of interest: (1) demographic and general farm information, (2) herd characteristics (e.g. herd size, number of lactating cows, number of heifers etc.), (3) reproduction management practices (use of bull or artificial insemination (AI), transfer of embryos, use of sexed semen etc.) and (4) feeding management (weaning age, number of different feeding groups, feed ingredients, diets etc.).

Three data sets were developed: (1) reproduction performance, (2) growth performance and (3) ration evaluation, hereafter referred to as reproduction, growth and ration data sets, respectively. Respective information

was collected by age group (weaning to calving) to provide an insight into both past and potential future performance and to estimate the current situation of NUE at the time of sampling.

To assess the reproduction performance, the developed dataset included records from 2453 Holstein cows from 12 herds. Data from two farms were not taken into consideration and consequently were excluded from the data set, due to inconsistent tracking of the reproduction scheme (e.g. missing dates of first service and first calving, number of services etc.). For the assessment of growth, 648 heifers from 13 herds of different age groups were included. One farm was excluded from the dataset, as on-farm conditions (lack of headlocks, slippery floor, behavioral problems) did not allow the measurement of a sufficient number of animals, making difficult to obtain accurate data. For the ration evaluation dataset, total mixed rations (TMR) from 10 herds were inputted into the Cornell Net Carbohydrate and Protein System (CNCPS; Van Amburgh et al., 2015). The TMR of 4 herds were not used for the following reasons: (1) two of them were excluded from growth dataset, (2) one due to the use of commercial compound feeds with unknown ingredient composition, as it was not provided by the manufacturer, and (3) one because heifers were fed lactating cows' refusals in a not consistent way, making it difficult to calculate the exact amount of each ingredient and the ratio between the ingredients included in the final TMR.

2.3.2. Feed sampling

A detailed description of the TMR was recorded before the feed sampling, so that all individual ingredients included in TMR could be sampled. Forage samples with high content of moisture (e.g. silages), were obtained from

the fresh cut face of the silo. At least 5 samples of 0.5 kg each were taken from different spots of the silo. Then, samples were placed in a bucket, thoroughly mixed and subsampled to reach 1 kg of weight. Samples were kept at -20 °C until further analysis. In the case of hay and straw or low moisture forages, samples were collected from the sections that were going to be fed the same day of the visit and the same procedure, as previously mentioned, was followed. Samples were stored at 4 °C until processing.

The concentrate feeds were sampled during the TMR preparation. In the case of a vertical silo, the sample collection took place while the feed ingredients were placed into the mixer wagon. In case of stockpiles, at least 5 samples of 0.5 kg each were taken from different spots of the stockpile. The sample was thoroughly mixed, subsampled to final weight of 0.5 kg and kept at 4 °C until analysis.

The total amount of TMR offered was recorded for each feeding group. In order to create a representative sample of TMR for each feeding group, at least 10 samples were collected from the feeding line, placed in a bucket, mixed and subsampled to final weight of 1 kg. All samples of TMRs were placed in sampling bags and were kept frozen at -20 °C until further analysis.

2.3.3. Animal measurements

All farms reared Holstein cattle. To evaluate the dairy heifer growth, mature body weight (MBW) of the herd was determined. For this, in each herd about 10-15% of lactating cows were randomly selected and the heart girth circumference (HGC) was measured using a measuring tape. The body weight of the cows was calculated using Yan's equation:

$$BW=6.373*HGC-662.6 \text{ (Yan et al., 2009),}$$

where BW in kg and HGC in cm. The upper quartile of the distribution of the body weights was used as MBW for each herd.

In each different growing stage, from weaning up to pre-calving, 15-20% of heifers were randomly selected and the HGC was recorded. It should be noted that HGC and BW are high correlated (Heinrichs et al., 2007, Swali et al., 2008, Bond et al., 2015). The HGC was measured using a measuring tape and animals' identification numbers were recorded in order to retrieve the dates of birth. The body weight of heifers was estimated by the following equation:

$$BW=65.36-(1.966*HGC) + (0.1959*HGC^2) + (0.00001691*HGC^3)$$

(Heinrichs et al., 1992), where BW in kg and HGC in cm.

2.3.4. *Reproduction performance*

For the evaluation of reproduction performance, a data set was created containing information regarding country of origin, date of birth, first service, number of services, first calving and use of bull or AI. Using Microsoft Excel for each herd, age at first service (T_{1stSer}), age at conception (T_{Conc}) and age at first calving (T_{1stCal}) was calculated and the number of services needed to conceive was determined. Moreover, the database was edited to remove any data-entry mistakes (e.g. pregnancy duration, based on date of breeding and calving) and until no outliers were observed.

2.3.5. *Growth performance*

Using Microsoft Excel, for each herd, regression equations were created, with the dependent and independent variables being the BW and age in days, respectively. Then, the one with the best fit, based on highest R^2 value,

was selected, which in all cases were linear. Thus, heifers' BW at a herd level was estimated at different time points of interest, which were at weaning age, T_{1stSer} and T_{1stCal} . In parallel, first service and first calving were previously calculated as an average of the herd. Hence, by using the regression equation, the respective BW was estimated (actual BW) for each farm. As an example, Figure 1 demonstrates the linear regression created for each herd (Fig. 2.1A) and for the entire population of the current study (Fig. 2.1B). Thus, it was allowed to calculate the average daily gain (ADG) for each individual growing stage, that is weaning, breeding, calving (ADG_{1stSer} , ADG_{SerCal} , ADG_{1stCal} , respectively) and to proceed with the evaluation of growth. According to National Research Council (NRC), growth targets (BW targets) based on each farm MBW were set as follows: (1) 55% MBW at first service ($Target_{55}$), (2) 85% MBW at calving ($Target_{85}$) and (3) 95% MBW at calving (NRC, 2001). Thus, the respective ADG targets for each growth stage were set. Then, it was examined if the way that heifers were growing was allowing them to reach the set BW targets, by predicting the BW (BW predicted) that heifers would have had at 14.3 and 24 months, which were the target time points. In other words, we investigated the potential of these animals to have been bred and calved at 14 and 24 months, respectively.

2.3.6. Ration evaluation

Evaluation of rations was performed using the CNCPS (AMTS platform, version 6.5; Van Amburgh et al., 2015). The TMR from all age groups, was input into the CNCPS and the evaluation was performed in 3-month intervals, beginning after weaning. Thus, the evaluation groups that were formed were these of 3, 6, 9, 12, 15, 18, 21 and 24 months of age. The model inputs that

were used included: the chemical analysis of the feeds, the TMR composition of each group, the age in months, the respective BW, MBW, ADG, the AFC and days of pregnancy. The following outputs from CNCPS were used: supplemented and required metabolizable protein (MP), supplemented and required metabolizable energy (ME), MP derived from bacteria (MP_{Bact}), rumen ammonia balance ($Rumen_{NH_3}$), productive N, rumen degradable protein (RDP) and rumen undegradable protein (RUP). Then, based on the productive N, NUE of each group was calculated ($NUE, \% = \text{productive N} * 100$).

In some cases, the formulated TMR was fed in multiple age groups regardless of the growth requirements of each stage. Moreover, it was difficult to determine dry matter intake (DMI) at the specific time points for each group, as different age heifers were reared in the same pen. In order to address this challenge, the ratio of the feeds in the TMR was maintained and DMI was adjusted to match the first limiting factor, that is ME or MP. In all cases, ME as the first limiting factor and DMI was modified to meet heifer's requirements for it. Moreover, in most cases, during the last two months of pregnancy, heifers were moved to dry cows' pens. Thus, the TMR were evaluated up to 7 months of pregnancy according to each herd's average T_{1stCal} , to avoid any negative impact of regrouping and commingling (Cook and Nordlund, 2004; Kerwin et al., 2023).

2.3.7. Chemical analysis

All forages, concentrate and TMR samples were analyzed to determine their chemical composition. For the determination of dry matter (DM), samples with high moisture content (e.g. silages, TMR) were dried in a forced-air oven at 55 °C for 48 hours, while feeds low in moisture (e.g. straw, hay) were dried

at 103 °C for 24 hours (AOAC, 2005). All samples were ground to pass 1 mm screen (Ultra centrifugal mill, Retsch, Type ZM 200). For ash determination, all samples were ashed in a muffle set at 550 °C for 6-8 hours (AOAC, 2005). The crude protein (CP) level was determined by Kjeldahl method (AOAC, 2005) and neutral detergent fiber (NDF) as described by Van Soest method (Van Soest et al., 1991) using heat stable α -amylase and corrected for the ash content. Ether extract (EE) was determined by Soxhlet method (AOAC, 2005), while starch by the enzymatic method as described by Hall, (2015), using the Megazyme KTSA kit, (Megazyme, Bray, Wicklow, Ireland).

2.3.8. Statistical analysis

Descriptive statistics were performed using JMP (v.13, SAS). For each variable of interest, the median, mean, standard deviation, minimum and maximum values were determined. For growth variables, the mixed model included the status (Actual, Predicted and Target) as fixed effect, while for nutritional variables the mixed model included the growth stage (3, 6, 9, 12, 15, 18, 21, and 24 months) as fixed effect. In both cases the farm was treated as the random effect.

2.4. Results

2.4.1. Reproduction

All farms were practicing artificial insemination (AI) as the main reproduction management technique. There was only 1 farm that was using exclusively a bull for heifers even though they were practicing AI for cows of the herd. Over the entire data set, four out of fourteen farms were maintaining bulls in the herd and used them in case of multiple returns (following 2nd or 3rd unsuccessful AI).

Regarding T_{1stCal} and T_{1stSer} , at the farm level there was a wide variation among results. Five farms demonstrated an average T_{1stCal} close to 24 months, two farms close to 25 months and five farms had T_{1stCal} close to 26,5 months (Figure 2.2A). Similarly, regarding T_{1stSer} four farms started breeding around 14 months of age, two farms close to 15 months, four farms close to 16 months and two farms at 17 months (Figure 2.2B). Table 1 provides cumulative results of the reproduction data set at an animal level, whereas Table 2 presents results at a farm level. As it is presented, T_{1stSer} was 15.4 months of age (± 2.14), ranging from 8.8 to 29.7 months. The minimum and maximum values of T_{Conc} were 8.8 to 33.2, while the average conception age was 16.0 months (Table 1). In order to conceive, heifers needed 1.54 services, with few animals required up to 7 services. Moreover, T_{1stCal} was 25.3 (± 2.65) months, ranging from 17.0 to 41.9 months of age.

2.4.2. Growth

At a farm level, BW_{1stSer} was 437.1 ± 31.4 kg, which was not different from the predicted at 14,3 months, but it was 34 kg heavier from the Target BW_{1stSer} , considering a target of 55 % MBW for heifers at first service (Table 3). Average daily gain at first service (ADG_{1stSer}) did not differ among the actual, the predicted and the Target₈₅ and Target₉₅ ($P = 0.57$). For the period from 1st service to 1st calving, in order to achieve 1st calving at 24 months, heifers need an ADG_{SerCal} of 0.756 kg/day or 1.033 kg/day for Target₈₅ or Target₉₅, respectively. In reality, heifers were growing with an ADG_{SerCal} of 0.875 kg/day, which was between the two set targets. At T_{1stCal} heifers achieved both targets actually weighing 692.8 kg exceeding 69 kg the Target₈₅ ($P = 0.001$). Overall, heifers from weaning to calving should have had an ADG_{1stCal} of 0.834 kg/day

to achieve calving at 24 months with 85% MBW, but in order to calve in a heavier BW_{1stCal} and achieve Target₉₅, should have been growing with 0.953 kg/day. Nonetheless, for the entire growing period, that is from weaning to calving, heifers had an ADG of 0.875 kg/day, that was not statistically different from the predicted ADG at 24 months.

2.4.3. Rations-Grouping

The number of different TMR fed to heifers varied from farm to farm, ranging from 1 to 4, according to growing stage's requirements. The majority of the farms (42.9%) offered only one TMR to all heifer groups, as shown in Figure 3. Three farms (21.4 %) fed 3 TMR, three farms (21.4 %) 2 different TMR and two farms (14.3 %) provided 4 TMR. It needs to be noted that physical grouping (Figure 4) based on age did not follow dietary groups, since most farms created groups according to specific management practices (e.g. available facilities, age of breeding, feeding equipment etc.).

2.4.4. Ration Evaluation

The overall chemical composition of TMR fed to dairy heifers is summarized in Table 4. As it is shown, DM of TMR was on average 58.9 % ranging from 36.9 to 89.3 %. It should be noted that the TMR containing the lower limit of this range was a high yielding cow group TMR and was fed to 9-month heifer group. Moreover, forage to concentrate (F:C) ratio was on average 71:29. The chemical components and the evaluation of the diets of each separate age group are reported in Table 5 and 6. In all groups, ME was the first limiting nutrient. In parallel, MP was supplied above heifers' requirements and was progressively increasing along with heifers age ($P < 0.0001$). Furthermore, MP deriving from bacteria reached on average 64.9 % MP, with

3-month group exhibiting the lowest values among all groups. Rumen ammonia balance (RumenNH₃) was on average 127.1 % and no difference was observed among groups. The NUE was higher in the first growing period from 3 to 12 months, (on average 26.1 %; ($P < 0.0001$)), and was decreased as the heifer age was increased. Lower values of NUE found in the second growing period, from 15 to 24 months, averaging 13.0 %.

2.5. Discussion

2.5.1. *Reproduction and growth*

A generally accepted goal of heifer management is first calving age at 24 months old, as it is considered more economically sustainable (Ettema and Santos, 2004; Mohd nor et al., 2012) with cows demonstrating higher lifetime milk production (Eastham et al., 2018). To achieve this at the herd level, breeding should start earlier than 15 months to include any reproduction failures, such as returns or embryo losses in early gestation (Le Cozler et al., 2008). In the current study the target was set at 14.3 months. However, except from biological maturity that is achieved with age, body size (BW and height) is also important (Wathes et al., 2014). Fox et al., (1999) suggested that BW target in T_{1stSer} should be 55% of MBW. In the current study, heifers were first bred at 15.4 months with a higher BW compared with Target₈₅ and Target₉₅. When the BW of these heifers at 14.3 months was estimated it was not different than Target₈₅ and Target₉₅, meaning that it was possible to bred heifers at 14.3 months of age. In addition, heifers had the proper ADG to reach Target₈₅, 0.875 kg/d, but in order to achieve Target₉₅ they should have grown in a higher rate. It is noted that ADG should be calculated taking into consideration each herd's genetic potential and selected frame size (Hoffman, 2017). However, Zanton

and Heinrichs, (2005) suggested that an ADG of 700-900 g/d in the period before puberty optimizes first lactation performance without compromising growth. Nonetheless, there was a delay of 1 month extending that way non-productive period. This delay might be attributed to the lack of observation (Donovan et al., 2003), meaning that maybe there is a problem either in estrus detection or the implementation of estrus synchronization protocols. Out of fourteen farms, eight observed visually the herd to detect any heifers in estrus, six had sensory collars, one used an estrus synchronization protocol, whereas two had a bull as estrus detector along with the visual observation. Another reason for this delay may be attributed to the lack of weight monitoring (Cue et al., 2012). It was common in all farms the absence of weighing scales, both for heifers and lactating cows. Moreover, no measuring tape was used by the farmers to estimate BW. Instead, common practice was to visually assess the BW of the heifers, leading to wrong decisions sometimes. Indeed, some animals were bred as young as 8.8 months of age (Table 1). Other practice, was to use a crafted line in the wall as a guide to distinguish animals ready to be bred. Overall, even though from a BW perspective it was possible to start breeding heifers at 14.3 months, this was not achieved. Thus, better management practices including the installation of weight scales and frequent monitoring of BW are needed to assure proper ADG and BW for the different growth benchmarks, such as T_{1stSer} and T_{1stCal} .

2.5.2. Diet Evaluation and grouping management

Even though adequate amounts of rumen degradable protein (RDP) are essential to maintain milk production and animal growth (Nichols et al., 2022), CP overfeeding, that has been practiced to overcome forage CP variation

(Firkins and Reynolds, 2005), may lead to excess of ruminally available N. As result, the excess N not used by the animal is excreted with feces and mainly with urine (Van Amburgh et al., 2015; Foskolos and Plomaritou, 2023). This practice is usually carried over in replacement heifer nutritional management, especially when lactating TMR are fed to heifer's groups, as a time and cost saving solution in TMR preparation. Indeed, in our study from fourteen farms visited, five farms were providing lactating cows TMR at least at 1 group of heifers. It is obvious that a formulated TMR to support high milk production, will exceed growth requirements. For example, in case of Farm 5, it was provided to 12 month and 15-month-old group, a lactating cow TMR with CP at 16.7 % DM. While this is considered within ration recommendations for a high producing cow, led to MP supply of 131 and 140% of requirements when fed to a 12 month and 15-month group, respectively. This nutritional strategy partially explains the higher values within the range of CP and MP in the data set. Moreover, it does not take into consideration that N retention reduces as the animal reaches maturity (Lobley, 1993). Hence, lactation cows TMR should be provided with caution and always depending on heifer growth stage, since except from the environmental issues that this practice arises, it also may increase the overall production cost.

As shown in Figures 3 and 4, nutritional and physical grouping in many cases were different and this management practice may have multiple impacts. For example, Farm 6 displayed excess physical grouping, creating 10 different groups. This is likely attributed to facility shortage and too many heifers kept as inventory. However, right before calving, for one-month period, heifers were moved to another pen, and kept with dry cows. Housing of heifers and cows

may have negative effects, as the first are usually the recipients of aggression (Soonberg et al., 2021). Moreover, out of fourteen farms, six (Farm 6 included) fed only one TMR to different age groups without taking into consideration each phase's requirements. Requirements of CP in growing animals are difficult to be defined, because of constant increasing of energy and growth demands (Hoffman, 2017). However, previous studies have shown that N retention is optimized in diets with a CP level of 12.0 to 15.0 % DM (Hoffman et al., 2001; Marini and Van Amburgh, 2005; Zanton and Heinrichs, 2009). Johansen et al., (2022) fed heifers from 8 to 20 months of age, diets that contained 9.8, 12.2, 14.6 and 17.0 % CP of DM and suggested that a level of CP 12.2 % DM is sufficient to support good rumen conditions while reducing N losses in all age groups. Similarly, Zhang et al., (2017) fed 9 to 11 month old heifers diets with CP at 10.2, 11.9 and 13.5 % DM. The higher levels of CP did not bring further improvement of N utilization, suggesting that 11.9% CP of DM was adequate. In our study, across all farms, CP levels ranged from 8.4 to 16.7 % DM, with an average of 12.6 % DM (Table 4). Nevertheless, any extreme value of the dataset was moderate when examined per age group, with CP concentration being within the range of recommendations in all groups (Table 5). However, CP concentration of the diet should only be used as an indicator because MP is a more accurate measure of protein feeding (Tedeschi et al., 2015; Foskolos and Plomaritou, 2023).

In this direction, we reported an excess of MP in many cases. As shown in Table 6, MP was supplied in excess, and there was a significant difference between the older groups (21 and 24 months) and the rest of groups. When the difference between supplemented and required MP (Δ MP) was expressed as

% of the MP supply, it was evident that a surplus of 30.9 % MP was provided to 21-month-old group versus 8.8 % surplus to 3 months old. Simultaneously, we reported similar CP levels among most age groups but decreasing NUE with age (from 31.5 to 10.7 at 3 and 24 months old, respectively). Similar values have been reported from other studies. Based on data presented by Marini and Van Amburgh, (2005), it was calculated a NUE of 15.9 to 22.4 % as N intake was decreasing from 203.5 to 87.6 g/d, respectively for young heifers. When 20 months old heifers were fed a diet with CP at 12.2 or 14.6 % DM the NUE resulted at 14.8 and 12.1 %, respectively (Johansen et al., 2022). According to National Academies of Science, Engineering, and Medicine (NASEM), from a physiological point of view, N requirements are lower, lean tissue deposition is decreased and feed intake increases as heifers reach first calving (NASEM, 2021).

By reducing CP levels, Rumen_{NH3} concentration might be limited. As a diet formulation practice it was suggested that Rumen_{NH3} should be between 110-120 % of requirements, since its shortage may cause detrimental effects (Foskolos and Plomaritou, 2023), while its surplus may lead to increased urinary N excretion (Huhtanen et al., 2008; Van Amburgh et al., 2015). In our dataset we observed feeding groups with low Rumen_{NH3} (min = 77 %) but also feeding groups with excess of Rumen_{NH3} (max = 167 %), while on average Rumen_{NH3} was 127.1 % of requirements. Thus, on average there is room for improvement by decreasing the amount of RDP but because we observed high variation among farms this should be implemented on a case by case basis.

2.6. Conclusions

In conclusion, it is highly recommended the frequent monitoring of heifers' BW to attain the body size that permits first calving around 24 months of age along with a diet that promotes growth, while minimizes N losses. The trend that NUE follows, allows the nutritional manipulation of the diets depending on growth periods. Hence, lower CP concentration of the diet in older heifers, may eliminate N losses and increase NUE. In other words, heifer ration formulation should allow the implementation of multi-gear nutritional plan that meets higher MP demands per kg of BW until 12 months old and prevents from negative outputs after first service. Moreover, nutritional mechanistic models that balance rations on MP basis allow precision feeding and prevent any potential nutrient losses through estimation tools. The high variation that was observed in the population examined, indicates that strategies should be implemented depending on the case, farm to farm, since the approach one plan fits all may not be a sustainable option.

2.7. References

- Association of Official Analytical Chemists (AOAC), 2005. Official Methods of Analysis of AOAC International. AOAC International, Gaithersburg, MD.
- Boulton, A., Rushton, J., and Wathes, D., 2015. The management and associated costs of rearing heifers on UK dairy farms from weaning to conception. Open Journal of Animal Sciences 5, 294-308. doi: <https://doi.org/10.4236/ojas.2015.53034>.

- Broderick, G.A., 2018. Review: Optimizing ruminant conversion of feed protein to human food protein. *Animal* 12, 1722-1734. doi: <https://doi.org/10.1017/S1751731117002592>.
- Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., van Vuuren, A.M., 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184-1196. doi: <https://doi.org/10.1017/S1751731110000911>.
- Cook, N.B., Nordlund, K.V., 2004. Behavioral needs of the transition cow and considerations for special needs facility design. *Veterinary Clinics of North America: Food Animal Practice* 20, 495-520. doi: <https://doi.org/10.1016/j.cvfa.2004.06.011>.
- Cue, R.I., Pietersma, D., Lefebvre, D., Lacroix, R., Wade, K., Pellerin, D., Passillé, A.-M.d., Rushen, J., 2012. Growth modeling of dairy heifers in Québec based on random regression. *Canadian Journal of Animal Science* 92, 33-47. doi: <https://doi.org/10.4141/cjas2011-083>.
- Donovan, G.A., Bennett, F.L., Springer, F.S., 2003. Factors associated with first service conception in artificially inseminated nulliparous Holstein heifers. *Theriogenology* 60, 67-75. doi: [https://doi.org/10.1016/S0093-691X\(02\)01296-7](https://doi.org/10.1016/S0093-691X(02)01296-7).
- Eastham, N.T., Coates, A., Cripps, P., Richardson, H., Smith, R., Oikonomou, G., 2018. Associations between age at first calving and subsequent lactation performance in UK Holstein and Holstein-Friesian dairy cows. *Plos One* 13, e0197764. doi: <https://doi.org/10.1371/journal.pone.0197764>.

- Ettema, J.F., Santos, J.E.P., 2004. Impact of age at calving on lactation, reproduction, health, and income in first-parity Holsteins on commercial farms. *Journal of Dairy Science* 87, 2730-2742. doi: [https://doi.org/10.3168/jds.S0022-0302\(04\)73400-1](https://doi.org/10.3168/jds.S0022-0302(04)73400-1).
- Firkins, J.L., Reynolds, C.K., 2005. Whole-animal nitrogen balance in cattle. In *Nitrogen and phosphorus nutrition of cattle: Reducing the environmental impact of cattle operations* (ed. Pfeffer, E., Hristov, A.N.), CAB International, Wallingford, UK, pp. 167–186. <https://doi.org/10.1079/9780851990132.0167>.
- Foskolos, A., Moorby, J.M., 2018. Evaluating lifetime nitrogen use efficiency of dairy cattle: A modelling approach. *Plos One* 13, e0201638. doi: <https://doi.org/10.1371/journal.pone.0201638>.
- Foskolos, A., Plomaritou, A., 2023. Guest editorial: Holistic Nutritional Management as a tool to mitigate nitrogen pollution from dairy cattle farming. *The Journal of Agricultural Science*, 1-17. doi: <https://doi.org/10.1017/S0021859623000254>.
- Fox, D.G., Van Amburgh, M.E., Tylutki, T.P., 1999. Predicting requirements for growth, maturity, and body reserves in dairy cattle. *Journal of Dairy Science* 82, 1968-1977. doi: [https://doi.org/10.3168/jds.S0022-0302\(99\)75433-0](https://doi.org/10.3168/jds.S0022-0302(99)75433-0).
- Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the Nitrogen Cycle: Recent Trends, Questions, and

Potential Solutions. Science 320, 889. doi:
<https://doi.org/10.1126/science.1136674>.

Hall, M.B., 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: collaborative study. Journal of AOAC International 98, 397-409. doi:
<https://doi.org/10.5740/jaoacint.15-012>.

Heinrichs, A.J., Rogers, G.W., Cooper, J.B., 1992. Predicting body weight and wither height in Holstein heifers using body measurements. Journal of Dairy Science 75, 3576-3581. doi: [https://doi.org/10.3168/jds.S0022-0302\(92\)78134-X](https://doi.org/10.3168/jds.S0022-0302(92)78134-X).

Hoffman, P.C., 2017. Feeding management of the dairy heifer from 4 months to calving. In Large Dairy Herd Management (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA p. 431-443. doi: <https://doi.org/10.3168/ldhm.0632>.

Hoffman, P.C., Esser, N.M., Bauman, L.M., Denzine, S.L., Engstrom, M., Chester-Jones, H., 2001. Short Communication: Effect of dietary protein on growth and nitrogen balance of Holstein heifers. Journal of Dairy Science 84, 843-847. doi: [https://doi.org/10.3168/jds.S0022-0302\(01\)74542-0](https://doi.org/10.3168/jds.S0022-0302(01)74542-0).

Huhtanen, P., Nousiainen, J.I., Rinne, M., Kytölä, K., Khalili, H., 2008. Utilization and partition of dietary nitrogen in dairy cows fed grass silage-based diets. Journal of Dairy Science 91, 3589-3599. doi:
<https://doi.org/10.3168/jds.2008-1181>.

- Johansen, M., Madsen, P.A., Brask-Pedersen, D.N., Lund, P., 2022. Effect of age and dietary crude protein level on nitrogen excretion in dairy heifers. *Livestock Science* 264, 105058. doi: <https://doi.org/10.1016/j.livsci.2022.105058>.
- Kerwin, A.L., Burhans, W.S., Nydam, D.V., Overton, T.R., 2023. Transition cow nutrition and management strategies of dairy herds in the northeastern United States: Part III—Associations of management and dietary factors with analytes, health, milk yield, and reproduction. *Journal of Dairy Science* 106, 1246-1266. doi: <https://doi.org/10.3168/jds.2022-21876>.
- Le Cozler, Y., Lollivier, V., Lacasse, P., Disenhaus, C., 2008. Rearing strategy and optimizing first-calving targets in dairy heifers: a review. *Animal* 2, 1393-1404. doi: <https://doi.org/10.1017/S1751731108002498>.
- Lobley, G.E., 1993. Species comparisons of tissue protein metabolism: effects of age and hormonal action. *The Journal of Nutrition* 123, 337-343. doi: https://doi.org/10.1093/jn/123.suppl_2.337.
- Marini, J.C., Van Amburgh, M.E., 2005. Partition of nitrogen excretion in urine and the feces of Holstein replacement heifers. *Journal of Dairy Science* 88, 1778-1784. doi: [https://doi.org/10.3168/jds.S0022-0302\(05\)72852-6](https://doi.org/10.3168/jds.S0022-0302(05)72852-6).
- Mohd Nor, N., Steeneveld, W., Mourits, M.C., Hogeveen, H., 2015. The optimal number of heifer calves to be reared as dairy replacements. *Journal of Dairy Science* 98, 861-871. doi: <https://doi.org/10.3168/jds.2014-8329>.
- Mohd nor, N., Steeneveld, W., Mourits, M.C.M., Hogeveen, H., 2012. Estimating the costs of rearing young dairy cattle in the Netherlands

- using a simulation model that accounts for uncertainty related to diseases. *Preventive Veterinary Medicine* 106, 214-224. doi: <https://doi.org/10.1016/j.prevetmed.2012.03.004>.
- Mohd Nor, N., Steeneveld, W., van Werven, T., Mourits, M.C.M., Hogeveen, H., 2013. First-calving age and first-lactation milk production on Dutch dairy farms. *Journal of Dairy Science* 96, 981-992. doi: <https://doi.org/10.3168/jds.2012-5741>.
- NASEM, 2021. Nutrient requirements of dairy cattle, 8th revised edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/25806>.
- Nichols, K., de Carvalho, I.P.C., Rauch, R., Martín-Tereso, J., 2022. Review: Unlocking the limitations of urea supply in ruminant diets by considering the natural mechanism of endogenous urea secretion. *Animal* 16, 100537. doi: <https://doi.org/10.1016/j.animal.2022.100537>.
- NRC, 2001. Nutrient requirements of dairy cattle, Seventh Revised Edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/9825>.
- Overton, M., Dhuyvetter, K., 2017. Economic considerations regarding the raising of dairy replacement heifers. In *Large dairy herd management* (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA, p. 457-474. doi: <https://doi.org/10.3168/ldhm.0632>.
- Siafakas, S., 2019. Investigation of the feeding cost of ruminants using home-grown and/or procured feedstuffs depending on the production system. PhD Dissertation, Agricultural University of Athens, Athens.

- Soonberg, M., Kass, M., Kaart, T., Barraclough, R., Haskell, M.J., Arney, D.R., 2021. Effect of grouping on behaviour of dairy heifers and cows in the transition period. *Journal of Dairy Research* 88, 45-51. doi: <https://doi.org/10.1017/S0022029921000066>.
- Sutton, M.A., van Grinsven, H., Billen, G., Bleeker, A., Bouwman, A.F., Bull, K., Erisman, J.W., Grennfelt, P., Grizzetti, B., Howard, C.M., Oenema, O., Spranger, T., Winiwarter, W., 2011. Summary for policy makers. In *The European Nitrogen Assessment: Sources, Effects and Policy Perspectives* (ed. Bleeker, A., Grizzetti, B., Howard, C.M., Billen, G., van Grinsven, H., Erisman, J.W., Sutton, M.A. and Grennfelt, P.), Cambridge University Press, Cambridge, UK, p. xxiv-xxxiv. doi: <https://doi.org/10.1017/CBO9780511976988.002>.
- Tedeschi, L.O., Fox, D.G., Fonseca, M.A., Cavalcanti, L.F.L., 2015. Models of protein and amino acid requirements for cattle. *Revista Brasileira de Zootecnia* 44, 109-132. doi: <https://doi.org/10.1590/S1806-92902015000300005>.
- Van Amburgh, M.E., Collao-Saenz, E.A., Higgs, R.J., Ross, D.A., Recktenwald, E.B., Raffrenato, E., Chase, L.E., Overton, T.R., Mills, J.K., Foskolos, A., 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. *Journal of Dairy Science* 98, 6361-6380. doi: <https://doi.org/10.3168/jds.2015-9378>.
- Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to

- animal nutrition. *Journal of Dairy Science* 74, 3583-3597. doi:
[https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2).
- Wathes, D.C., Pollott, G.E., Johnson, K.F., Richardson, H., Cooke, J.S., 2014. Heifer fertility and carry over consequences for life time production in dairy and beef cattle. *Animal* 8, 91-104. doi:
<https://doi.org/10.1017/S1751731114000755>.
- Yan, T., Mayne, C.S., Patterson, D.C., Agnew, R.E., 2009. Prediction of body weight and empty body composition using body size measurements in lactating dairy cows. *Livestock Science* 124, 233-241. doi:
<https://doi.org/10.1016/j.livsci.2009.02.003>.
- Zanton, G.I., Heinrichs, A.J., 2005. Meta-Analysis to assess effect of prepubertal average daily gain of Holstein heifers on first-lactation production. *Journal of Dairy Science* 88, 3860-3867. doi:
[https://doi.org/10.3168/jds.S0022-0302\(05\)73071-X](https://doi.org/10.3168/jds.S0022-0302(05)73071-X).
- Zanton, G.I., Heinrichs, A.J., 2009. Digestion and nitrogen utilization in dairy heifers limit-fed a low or high forage ration at four levels of nitrogen intake¹. *Journal of Dairy Science* 92, 2078-2094. doi:
<https://doi.org/10.3168/jds.2008-1712>.
- Zhang, B., Wang, C., Liu, H., Liu, J., Liu, H., 2017. Effects of dietary protein level on growth performance and nitrogen excretion of dairy heifers. *Asian-Australasian Journal of Animal Sciences* 30, 386-391. doi:
<https://doi.org/10.5713/ajas.16.0214>.

2.8. Tables and Figures

Table 2.1. Descriptive statistics of the reproduction performance dataset, including individual replacement heifer records.

Item ¹	Mean	SD	Min	Max
T _{1stSer} , months	15.4	2.1	8.8	29.7
T _{Conc} , months	16.0	2.7	8.8	33.2
T _{1stCal} , months	25.3	2.7	17.0	41.9
No. of services	1.5	1.0	1.0	7.0

¹T_{1stSer} = time of first service; T_{Conc} = time of conception; T_{1stCal} = time of first calving

Table 2.2. Time of first service (T_{1stSer}), conception (T_{Conc}), first calving (T_{1stCal}) and number of services of replacement heifers per farm.

Farm	T _{1stSer} , mo	SD	T _{Conc} , mo	SD	No. Ser	SD	T _{1stCal} , mo	SD
1	16.1	0.2	16.7	0.2	1.4	0.1	26.0	0.2
2	17.2	0.2	17.3	0.3	1.1	0.1	26.7	0.3
3	16.4	0.1	17.6	0.1	1.8	0.1	26.7	0.2
4	16.9	0.2	17.9	0.2	1.4	0.1	26.8	0.2
5	16.4	0.2	18.0	0.2	2.1	0.1	27.0	0.3
6	15.1	0.2	16.3	0.2	1.9	0.1	25.1	0.2
7	15.8	0.2	16.3	0.2	1.4	0.1	25.1	0.2
8	14.1	0.1	14.4	0.2	1.3	0.1	24.4	0.2
9	14.6	0.1	15.2	0.2	1.6	0.1	24.5	0.2
10	15.2	0.1	15.2	0.1	1.0	0.1	24.6	0.1
11	14.5	0.1	15.3	0.2	1.9	0.1	24.4	0.2
12	14.4	0.2	15.4	0.2	2.0	0.1	24.2	0.3

Table 2.3. Body weight (BW) and average daily gain (ADG) of replacement heifers, at first service (1stSer) and first calving (1stCal) compared to the predicted values at 14,3 and 24 months setting targets at first service (55 % of mature BW) and at calving at 85% or 95% of mature BW.

Item ¹	Actual	Predicted	Target	SEM	P-value
1st service 55% MBW					
BW _{1stSer} , kg	437.1 ^a	407.9 ^{ab}	403.4 ^b	8.7	0.02
ADG _{1stSer} , kg/day	0.875	0.875	0.899	0.020	0.57
1st Calving 85% MBW					
BW _{1stCal} , kg	692.8 ^a	662.5 ^{ab}	623.4 ^b	14.0	0.001
ADG _{SerCal} , kg/day	0.875 ^b	0.875 ^b	0.756 ^c	0.021	< 0.001
ADG _{1stCal} , kg/day	0.875 ^b	0.875 ^b	0.834 ^b	0.019	< 0.001
1st Calving 95% MBW					
BW _{1stCal} , kg	692.8 ^a	662.5 ^{ab}	696.7 ^a	14.0	0.001
ADG _{SerCal} , kg/day	0.875 ^b	0.875 ^b	1.033 ^a	0.021	< 0.001
ADG _{1stCal} , kg/day	0.875 ^b	0.875 ^b	0.953 ^a	0.019	< 0.001

^{a-c} Within a row means without a common superscript differ (P < 0. 05).

¹ BW_{1stSer} = BW at first service; ADG_{1stSer} = ADG from weaning to first service;
 BW_{1stCal} = BW at first calving; ADG_{SerCal} = ADG from first service to first calving;
 ADG_{1stCal} = ADG from weaning to calving.

Table 2.4. Descriptive statistics of the diet evaluation dataset, regarding replacement heifers' rations.

Item ¹	Mean	SD	Min	Max
DM, %	55.8	10.7	36.9	89.3
Forage DM, % of total DM	70.7	13.3	38.2	91.7
CP, % DM	12.9	2.2	8.4	16.7
aNDFom, % DM	47.8	9.2	28.3	66.7
Starch, % DM	18.2	5.2	8.7	28.2
Sugars, % DM	4.1	1.5	2.2	8.7
EE, % DM	2.9	0.6	2.1	4.8

¹DM = dry matter; CP = crude protein; aNDFom = neutral detergent fiber

determined with the use of heat stable α -amylase and corrected for ash

content; EE = ether extract

Table 2.5. Chemical composition of replacement heifer rations at different age groups.

¹ Item	Age groups								SEM	P-value
	3	6	9	12	15	18	21	24		
DM, %	61.9	60.7	54.5	54.4	55.1	54.8	54.7	58.5	3.5	0.11
Forage DM, % of total DM	56.5 ^c	60.4 ^{bc}	70.6 ^{ab}	73.5 ^a	74.9 ^a	74.5 ^a	75.3 ^a	75.4 ^a	3.8	<0.0001
CP, % DM	14.6 ^a	14.3 ^{ab}	12.9 ^{abc}	12.7 ^{bc}	12.2 ^c	12.6 ^c	12.4 ^c	12.4 ^c	0.7	0.0001
aNDFom, % DM	38.4 ^b	40.4 ^b	47.9 ^a	48.5 ^a	49.9 ^a	49.5 ^a	50.0 ^a	49.9 ^a	2.8	<0.0001
Starch, % DM	21.1 ^a	20.2 ^a	18.1 ^a	17.6 ^a	17.4 ^a	17.6 ^a	17.4 ^a	17.5 ^a	1.7	0.21
Sugars, % DM	5.3 ^a	5.1 ^a	3.8 ^a	3.8 ^a	3.8 ^a	3.6 ^a	4.2 ^a	3.5 ^a	0.5	0.02
EE, % DM	3.4 ^a	3.3 ^{ab}	2.9 ^{ab}	2.8 ^{ab}	2.7 ^b	2.8 ^b	2.7 ^b	2.7 ^b	0.2	0.001
RDP, % CP	63.4 ^c	67.2 ^b	67.9 ^{ab}	69.0 ^a	68.8 ^a	69.0 ^a	69.0 ^a	68.7 ^{ab}	1.0	<0.0001
RUP, % CP	36.6 ^a	32.4 ^b	32.1 ^b	31.0 ^b	31.2 ^b	31.0 ^b	31.0 ^b	31.3 ^b	1.0	<0.0001

^{a-g} Within a row means without a common superscript differ (P < 0. 05).

¹ DM = dry matter; CP = crude protein; aNDFom = neutral detergent fiber determined with the use of heat stable α -amylase corrected for the ash content; EE = ether extract; RDP = rumen degradable protein (CNCPS calculated); RUP = rumen undegradable protein (CNCPS calculated).

Table 2.6. Diet characteristics evaluation of replacement heifer rations using the Cornell Net Carbohydrate and Protein System at different age groups.

¹ Item	Age groups								SEM	P-value
	3	6	9	12	15	18	21	24		
DMI, kg/d	4.4 ^f	5.6 ^f	7.4 ^e	8.9 ^d	10.8 ^c	12.1 ^c	13.6 ^b	15.2 ^a	0.6	<.0001
ME, % req	101	103	103	101	100	101	100	101	1	0.19
ME _{av} , Mcal/d	9.8 ^h	12.5 ^g	16.2 ^f	19.4 ^e	23.1 ^d	26.2 ^c	29.4 ^b	32.9 ^a	1.0	<.0001
MP, % req	110 ^{de}	103 ^e	109 ^{de}	117 ^{cd}	121 ^{bc}	129 ^{ab}	133 ^a	133 ^a	3	<.0001
MP, g/d	441 ^g	528 ^g	661 ^f	782 ^e	925 ^d	1056 ^c	1177 ^b	1323 ^a	53	<.0001
ΔMP, g/d	44 ^e	14 ^{de}	53 ^{de}	112 ^{cd}	165 ^c	239 ^b	287 ^{ab}	319 ^a	26	<.0001
MP Bact, g/d	247 ^h	328 ^g	430 ^f	519 ^e	613 ^d	696 ^c	782 ^b	876 ^a	24	<.0001
MP Bact, % MP	57.6 ^c	62.3 ^b	65.4 ^{ab}	67.0 ^a	67.2 ^a	66.7 ^a	67.2 ^a	67.0 ^a	1.8	<.0001
Rumen NH ₃ , % req	132	133	129	127	126	128	126	125	7	0.64
NUE, %	31.4 ^a	30.5 ^a	23.7 ^b	19.5 ^c	16.3 ^d	13.6 ^e	11.8 ^{ef}	10.9 ^f	1.2	<.0001

^{a-g} Within a row means without a common superscript differ (P < 0. 05).

¹DMI = DM intake; MP = metabolizable protein; ME = metabolizable energy; MEav = available metabolizable energy; Δ MP = difference between supplemented and required MP; MP Bact = MP deriving from rumen bacteria; Rumen NH_{3Req} = Rumen ammonia expressed as a percentage of requirements; NUE = nitrogen use efficiency.

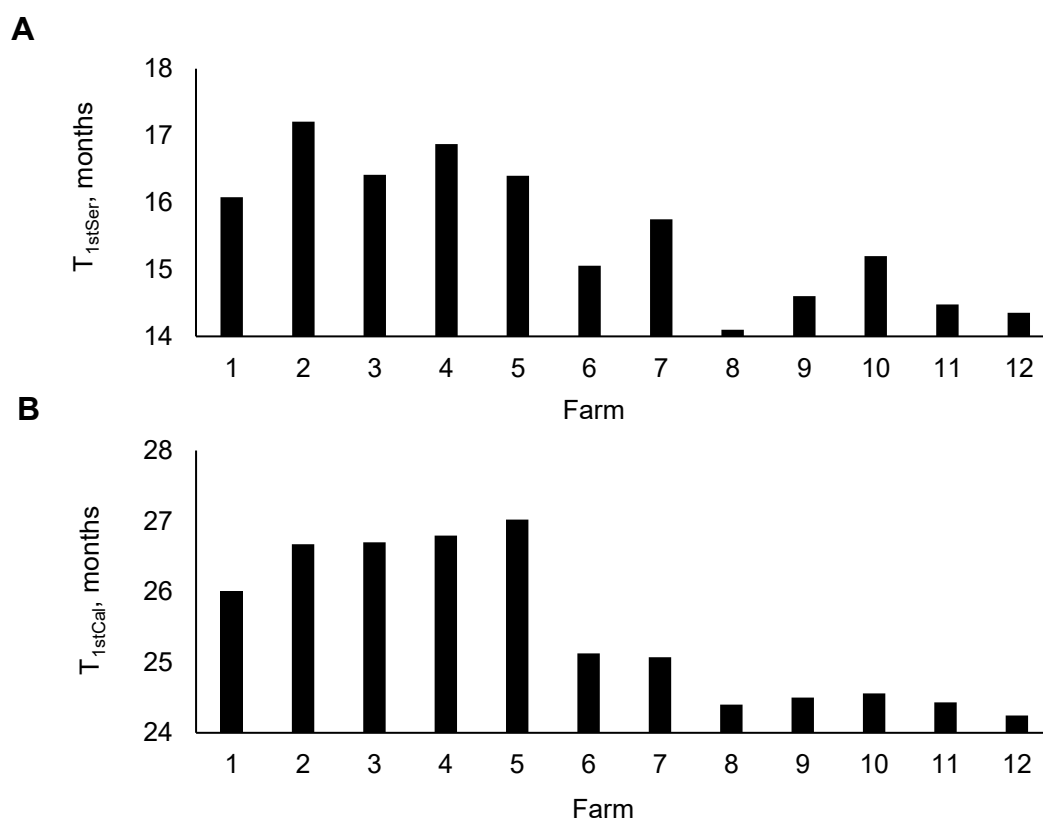


Figure 2.1. Time of: A: first calving (T_{1stCal}) and B: first service (T_{1stSer}) of dairy replacement heifers in different farms.

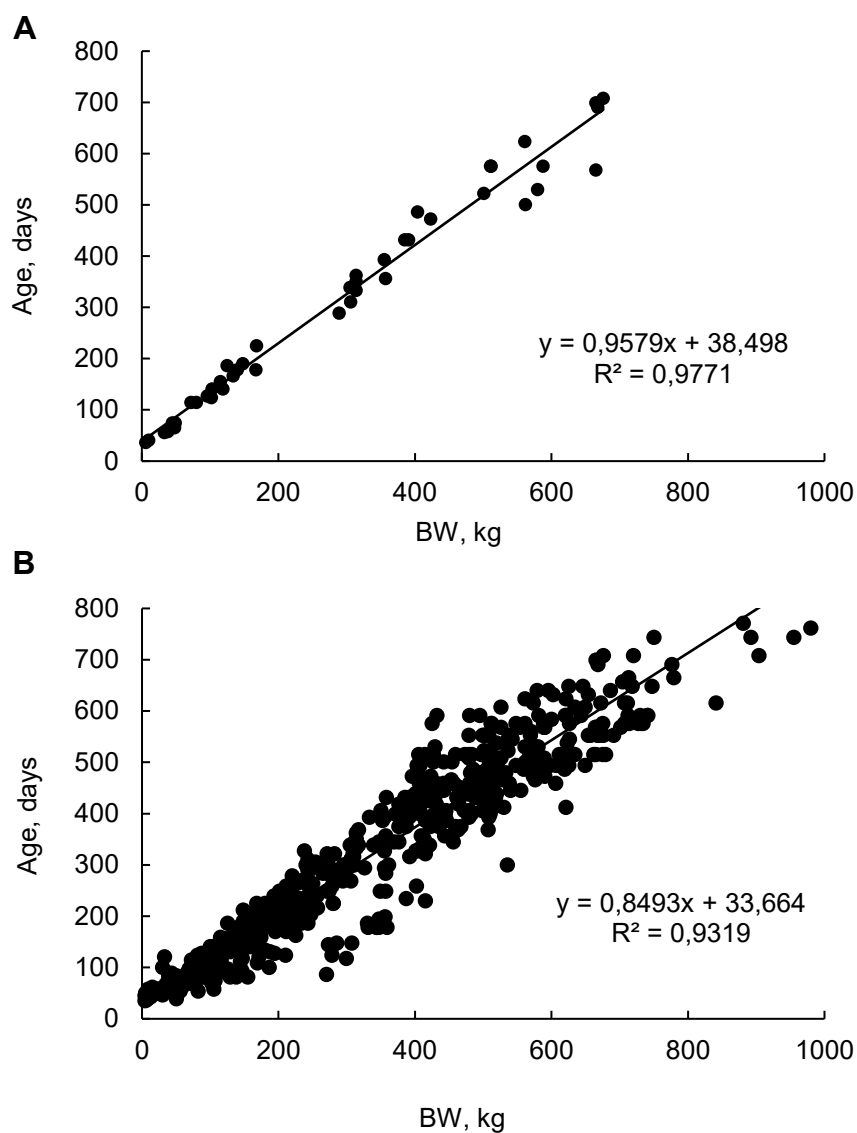


Figure 2.2. Body weight (BW) prediction equations of Holstein heifers (A) at the farm level and (B) for the whole population of heifers measured.

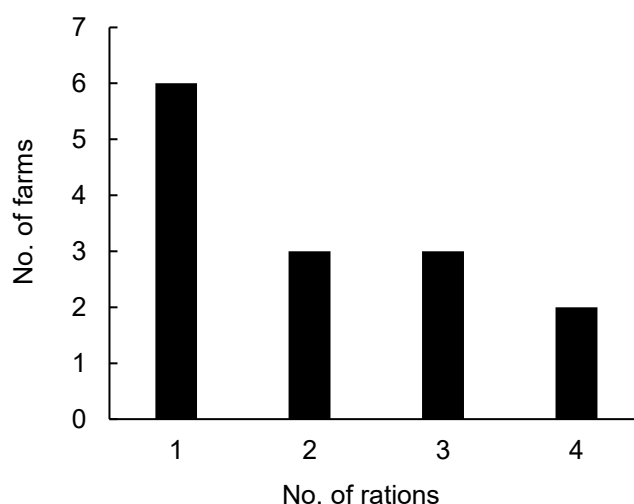


Figure 2.3. Number of different rations fed to dairy replacement heifers.

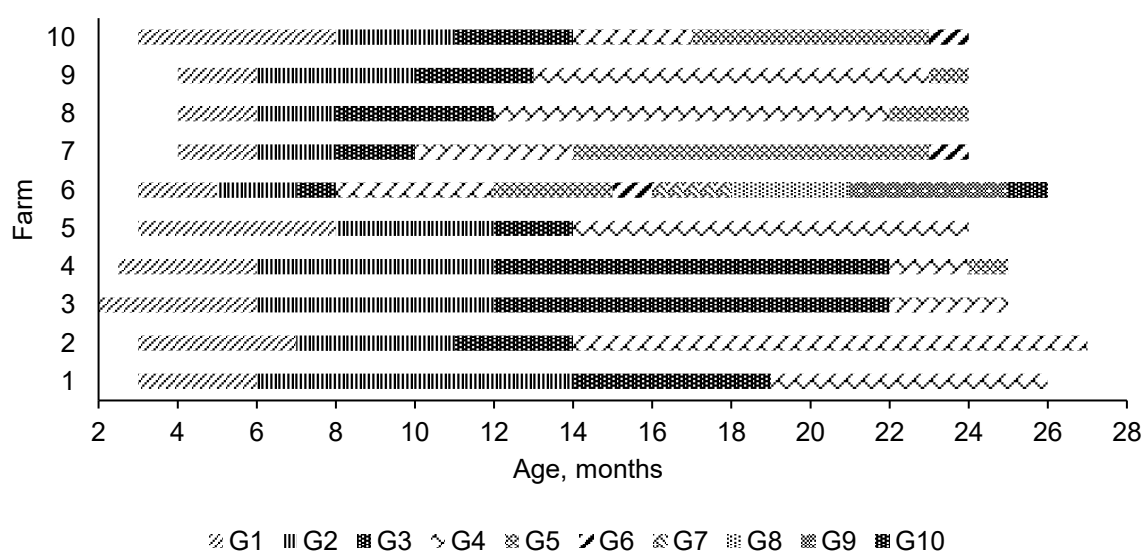


Figure 2.4. Physical on-farm grouping of replacement heifers. Different patterns stand for different groups

2.9. Supplementary material

Questionnaire used to obtain information about overall farm and replacement heifer management.

Data collection questionnaire

Table 1. Farm information.

A. Contact information

1.1. First and last name

1.2. Phone number

1.3. Address

1.4. Visit date

B. Animals

2.1. Dairy cows number

2.2. Replacement heifer number

2.3. Calvings/year

2.4. Female percentage (of 2.3)

C. Cow and heifer reproduction management

3.1. Use of AI (Yes/No)

3.2. Use of bull (Yes/No)

3.3. If yes, what's the percentage
of total cows

3.4. Use of sexed semen (Yes/No)

3.5. If yes, what's the percentage

of total cows

3.6. Use of embryos (Yes/No)

3.7. If yes, what's the percentage

of total cows

D. Heifer nutritional management

4.1. Weaning age

4.2. Group number with different

ration

4.3. Group description

4.4. Heifer ration number

Table 2. Ration description.

	Ration 1	Ration 2	Ration 3	Ration 4
Feeds	(kg/ mix)	(kg/ mix)	(kg/ mix)	(kg/ mix)

Table 3. Description of compound feeds/mixes

	Composition	Chemical composition	
Feed	%	%	
		Protein	
		Carbohydrates	
		Fat	
		Fiber	

Table 4. Replacement heifer information

Animal code	Birth date	Weaning date	BW	BCS

Table 5. Dairy cow information

(Cal=calving, DP=dry period)

Animal code	Birth date	1 st Cal date	1 st DP date	2 nd Cal date	2 nd DP date	3 rd Cal date	3 rd DP date

Chapter 3

Digestibility trials: Effects of two crude protein and rumen degradable protein levels on the apparent digestibility and nitrogen use efficiency of rations fed to dairy replacement heifers

3.1. Abstract

The objective of the study was to examine the effect of CP and RDP levels on intake, nutrient digestibility and N use efficiency (NUE) of dairy replacement heifers. The study comprised of 2 trials in a switchback design. Each trial lasted 3 experimental periods with 2 treatments in 8 heifers ($n = 8$). In first trial 2 levels of CP were offered to heifers, one high (14.9 % DM; HCP) and one low (12.1 % DM; LCP) in CP. In the second trial, offered diets contained two different RDP levels, one high at 75.2 % CP (HRDP) and one low at 64.3 % CP (LRDP) in a LCP diet. Heifers were housed individually. During sampling period, we recorded offer and refusals weight and we collected offer, refusal, feed, feces, urine, rumen fluid and blood samples. In first trial, N intake was higher in HCP compared with LCP treatment (164.9 vs 130.4 g/d, respectively, $P < 0.01$). The urinary N was higher for HCP heifers (60.8 and 43.1 g/d for HCP and LCP, respectively, $P < 0.01$). The higher N intake had corresponding effects on rumen $\text{NH}_3\text{-N}$ (12.6 and 8.1 mg/dL for HCP and LCP, respectively, $P = 0.03$) and blood urea levels (4.8 and 3.3 mmol/L for HCP and LCP, respectively, $P < 0.01$). The N balance was higher for HCP than LCP heifers (48.3 and 34.8 g/d for HCP and LCP respectively, $P = 0.03$), however we observed no effect of the higher CP level on NUE. In second trial, N intake was not affected, when the RDP level was altered. Rumen $\text{NH}_3\text{-N}$ was higher for HRDP compared with LRDP treatment (10.9 and 4.5 mg/dL for HRDP and LRDP, respectively, $P < 0.01$). Correspondingly, blood urea N was greater for HRDP heifers (5.4 and 3.7 mmol/L for HRDP and LRDP, respectively, $P < 0.01$). We observed no difference on N excretion and N balance. However, a tendency for decreased NUE was observed for heifers fed the HRDP treatment (17.4 and 23.0 % for

HRDP and LRDP, respectively, $P = 0.10$). In conclusion, even though higher CP level affected positively N balance, higher N concentration in rumen was observed, leading to increased N excretion in the environment. At a constant lower CP level and lower RDP protein level appears to reduce N losses in the environment via urine.

3.2. Introduction

Nitrogen (N) is an essential element to sustain life, however, its accumulation in different ecosystems has caused a series of polluting phenomena. The increased flow of anthropogenic N into the ecosystems, through the extended legume cultivation, fuel combustion and the Haber-Bosch process for the NH_3 production created unintended reactions of N in the ecosystems (Galloway et al., 2003; Sutton et al., 2021). Once the N cycle was disrupted, it led to multiple negative environmental effects (e.g. production of greenhouse gases, air quality degradation, soil and water acidification, eutrophication), a phenomenon described as N cascade (Galloway et al., 2003). Dairy cattle farming contributes to N flows through the fertilizer application on croplands and grasslands and through manure management (Dijkstra et al., 2013). It has been calculated that in the European Union (EU) 3.0 Tg of N_r enter the ecosystem to cover feed requirements of dairy cattle, while 2.2 Tg of N_r are excreted in feces and urine in each lactating period (Foskolos and Plomaritou, 2023). The amount N contained in the urine and excreted in the environment affects the amount of NH_3 emissions (Hristov et al., 2011). Urea contained in urine at a percentage of 50-90 % is rapidly hydrolyzed by urease presented in feces towards NH_3 formation (Bussink and Oenema, 1998). The volatilization rate of NH_3 is depended mainly on urinary N

concentration, as N contained in feces is organic bound and exhibits low volatilization rate (Hristov et al., 2011). The primary driver that affects N excretion is considered the N intake, with surplus N not utilized in milk or body N accretion being excreted in the environment mainly in the form of urine, but also with feces (Van Amburgh et al., 2015). A key action towards minimizing NH_3 losses is the increase of nitrogen use efficiency (NUE; Sutton et al., 2011). The NUE is a fraction between system's output N divided by system's input N (Erisman et al., 2018). Most efforts in increasing NUE focused on lactating herd by determining milk NUE (MNE; $\text{MNE, \%} = (\text{g of N in milk} / \text{g of dietary N}) * 100$); Calsamiglia et al., 2010), with calves and heifers receiving less attention. Adapting the logic at a heifer level, an increase in NUE can be achieved either by increasing the N retained in the body, while N intake remains the same or by decreasing the N intake without compromising growth.

Heifers require protein, and especially amino acids (AA), to support growth and maintenance (NASEM, 2021). Heifer protein requirements are not a constant number, but change dynamically until heifers reach maturity (Van Amburgh et al., 2019). Based on NASEM (2021), after puberty heifers with an average daily gain (ADG) of 840 g/day and mature body weight (MBW) at 700 kg, require a diet with CP content of 13.4 % DM. However, previous studies indicated that N retention is optimized in diets with a CP level from 12 to 15 % DM (Hoffman et al., 2001; Marini and Van Amburgh, 2005; Zanton and Heinrichs, 2009). Johansen et al. (2022) offered heifers of 8 to 20 months old, four levels of CP at 9.8, 12.2, 14.6 and 17.0 % DM and concluded that the CP level of 12.2 % DM supported growth in all age groups, while N losses were

minimized. Therefore, a lower CP level might benefit N utilization without compromising growth.

Of course, CP concentration of the diet alone does not reflect the accurate level of protein supply, as it does not provide any information about metabolizable protein (MP) content of the diet (Tedeschi et al., 2015; Foskolos and Plomaritou, 2023). The microbial protein and rumen undegradable protein (RUP) provide additional AA that are later ingested in the small intestine and consist the MP available to the heifer (Huhtanen and Ahvenjärvi, 2022). There is evidence that changes in ratio between rumen degradable protein (RDP) and rumen undegradable protein (RUP) may further improve N utilization and thus NUE (Davidson et al., 2003). Silva et al. (2018) examined the effects of RUP levels on N utilization and observed that increased levels of RUP from 48.9 to 54.0 % of CP, increased N retention and decreased N losses without affecting N intake. Thus, protein degradability and the amount of AA reaching the intestine hold an important role on N utilization.

Therefore, we hypothesized that: (1) urinary N excretion increases, while NUE decreases when the dietary CP level increases and (2) urinary N excretion decreases, while NUE increases when the RDP content of the diet decreases.

3.3. Material and methods

3.3.1. Animals and experimental design

The experimental procedures followed and the use of animals were approved by the Animal Care and Experimental Use Committee of University of Thessaly (Prot. N.45-130223). The study consisted of two digestibility experimental trials and was performed in a commercial farm. Eight Holstein

heifers averaging 335.1 ± 14.7 days, with initial BW of 259.8 ± 25.6 kg and BCS 3.5 ± 0.6 in Trial 1, and eight Holstein heifers averaging 410.1 ± 14.7 days, with initial BW 339.5 ± 28.6 kg and BCS 3.6 ± 0.6 in Trial 2 were enrolled in a switchback design with three experimental periods. Each experimental period lasted 25 days, with the first 21 days being used as adaption period, followed by a sampling period of 4 days. At the beginning of Trial 1, heifers were randomly assigned to one of two dietary treatments: a high crude protein (HCP; CP at 14.9 % DM) diet and a low crude protein (LCP; CP at 12.1 % DM) diet. Similarly, in Trial 2, maintaining the low CP level of Trial 1 in the two dietary treatments, heifers were randomly assigned either to a high rumen degradable protein (RDP; HRDP; RDP at 75.2 % CP) diet or a low rumen degradable protein (LRDP; RDP at 64.3 % CP) diet. The inclusion of forages and concentrates in a DM basis of the treatments is presented in Table 3.1 and 3.2 for Trial 1 and Trial 2, respectively, while the chemical composition of ingredients is shown in Table 3.3. In both trials, dietary treatments were isoenergetic. Heifers were housed individually and a total mixed ration (TMR) were offered ad libitum, once per day. Adjustments in the amount of feed offered were made daily to allow ad libitum feeding (5% refusal allowance). Rations were balanced to allow an ADG of 0.85 kg/d. The diet formulation was realized using the Cornell Net Carbohydrate and Protein System (CNCPS; Van Amburgh et al., 2015).

3.3.2. Sample collection and processing

3.3.2.1. Feed sampling

Individual feed ingredients included in the TMR were sampled daily during the four days of the sampling period. By the end of each period, feed

ingredient samples were mixed to make a representative composite sample for further analysis. Feed samples with high moisture content (e.g. corn silage) were kept at -20°C until further analysis, while feed samples with low moisture content (e.g. wheat straw, alfalfa hay, concentrates) were kept at 4°C until further analysis.

3.3.2.2. *Total tract apparent digestibility and nitrogen balance*

For the determination of total tract apparent digestibility TMR offer, TMR refusals and feces were sampled. For each heifer, the exact amount of TMR offered was recorded for four days and a representative sample was collected daily. Moreover, refusals of each heifer were collected and weighed. To form a representative sample for each heifer, TMR offer samples and the total amount of TMR refusals of all days, were dried in an air-forced oven set at 55°C, ground to pass a 5 mm screen and then mixed thoroughly to obtain a subsample equal with the 25% of the TMR offer sample and total TMR refusal amount. Then, the subsample was ground to pass 1mm screen and 200 gr were kept as the final TMR offer and TMR refusal sample for each heifer. At the end of the sampling period, offer and refusal samples were pooled respectively to form a representative composite sample and kept at -20°C for further analysis. Fecal samples were collected for three consecutive days to represent the 24h digestion cycle. Sampling took place four times per day with a 2h interval and a swift of 8h the following day. Fecal samples for each heifer were obtained directly from the rectum or by voluntary elimination, placed in a bucket and mixed. Then, individual fecal samples were placed into containers and kept in freezer until the end of the sampling period. At the end of the sampling period, samples were pooled per day and heifer (160 gr subsample from each time

point) to form a representative sample per heifer. The subsamples of all days were placed in a bucket and thoroughly mixed for 2 minutes using a mixer (Mixer 6000, Krausmann Power Tools, Athens, Greece) and increasing speed over mixing time. Then, a sample of approximately 1 kg was formed for each heifer and kept at -20°C until further analysis. Additionally, for the purpose of N balance determination urine sampling was realized. The sampling of urine was realized once a day with 8h intervals and for three consecutive days to represent the 24h digestion cycle. Urine sample from each heifer was collected after voluntary urination or by manual agitation of lower vulva. The sample was placed into plastic vessel, mixed thoroughly and divided into two aliquots. The aliquots included: (1) an aliquot of 50 mL was placed into plastic urine container with the addition of 1 mL of sulfuric acid (50%) to reduce pH<2 and was analyzed for N content and (2) an aliquot of 50 mL was kept without addition of acid for creatinine analysis. All urine samples were kept at -20°C until analysis.

3.3.2.3. *Heifer measurements*

The heifers' body weight (BW) was estimated by measuring the heart girth circumference (HGC) using a metric tape (Heinrichs et al., 1992) at the end of each sampling period. The body condition score (BCS) was recorded based on 5-point scale (1 = thin, 5 = obese) with 0.25 increments according to Edmonson et al. (1989).

3.3.2.4. *Rumen fluid and blood sampling*

At the last day of each sampling period, rumen fluid and blood samples were obtained from each heifer. The collection was realized using an oral tube (Ruminator; Profs Products, Wittibreut, Germany) attached to a vacuum pump, 4h after the delivery of TMR (Barros et al., 2019; Guo et al., 2019). The pH of

the fluid was immediately measured and 4 ml aliquots were placed in Falcon tubes, containing 4 ml of hydrochloric acid (0.2N). Rumen fluid samples were kept at -20°C until further analysis. Blood samples were collected through coccygeal vein into lithium heparin, 4h after feeding (Barros et al., 2019; Guo et al., 2019). Right after collection, lithium heparin vacutainers were centrifuged at 1500xg for 15 minutes at 4°C and then kept at -20°C until further analysis.

3.3.3. Chemical analysis

All samples of forages, concentrate feeds, TMR and refusals were analyzed to determine their chemical composition. For the determination of dry matter (DM), feed samples with high moisture content (e.g. silages) and feces were dried in a forced-air oven at 55 °C for 48 hours, while feeds low in moisture (e.g. straw, hay) were dried at 103 °C for 24 hours (AOAC, 2005). All samples were ground to pass 1 mm screen (Ultra centrifugal mill, Retsch, Type ZM 200). Samples of TMR offer and refusals were dried in a forced-air oven at 55 °C for 48 hours, as previously mentioned. All feed, TMR and fecal samples were analyzed for ash content at a muffle set at 550 °C for 6-8 hours (AOAC, 2005). The CP level was determined by Kjeldahl method (AOAC, 2005) and starch was determined by the enzymatic method as described by Hall, (2015), using the Megazyme KTSA kit (Megazyme, Bray, Wicklow, Ireland). The content of NDF was determined as described by Van Soest method (Van Soest et al., 1991) using heat stable α -amylase and corrected for the ash content (aNDFom).

For the determination of undigested NDF (uNDF) content in TMR offered and refused and dried feces, samples were incubated in duplicate using in vitro fermentation technique for 240 hours. The rumen fluid was obtained before

morning feeding from three cows using an oral tube (Ruminator; Profs Products, Wittibreut, Germany) attached to a vacuum pump. The cows were being fed on DM basis: corn silage (52.4 %), alfalfa hay (7.9 %), wheat straw (5.4 %), corn (14.9 %), soybean meal (13.1 %), wheat bran (6.2 %) and minerals and vitamins mix (0.3 %). Rumen fluid of each cow was placed into plastic thermos flasks and immediately transferred to the laboratory. Equal aliquots of each rumen fluid were strained through double layer of muslin under anaerobic conditions at 39 °C, thoroughly mixed and then used as an inoculant. After fermentation aNDFom content of samples was determined as described by Raffrenato et al. (2018).

Urine was analyzed for N content by the Kjeldahl method (AOAC, 2005). Creatinine content in urine and plasma urea N, urea, creatinine, albumin, BHBA and NEFA concentrations in blood were determined by spectrophotometric method on a biochemical analyzer (Siemens Dimension EXL 200 Integrated Chemistry System, Siemens; Vet in progress Biochemical Lab, Athens). Rumen fluid samples were thawed in a room temperature, centrifuged at 1700 x g for 20 minutes and used for the determination of ammonia-N concentration as described by Chaney and Marbach (1962).

3.3.4. Calculation of digestibility and nitrogen balance

Using uNDF as a marker, the fecal flow of DM ($\text{Flow}_{\text{FcDM}}$) was calculated as follows: $\text{Flow}_{\text{FcDM}}, \text{ kg/d} = (\text{uNDF}_{\text{int}}, \text{ kg} / (\text{uNDF}_{\text{Fc}}, \% \text{DM})/100)$. Then, component flows were calculated as follows: $\text{Flow (kg)} = (\text{Flow}_{\text{FcDM}} * \text{Component}_{\text{Fc}}/100)$. The total tract apparent digestibility of each component (DM, N, Starch and aNDFom) was calculated as follows: $\text{Digestibility (\%)} = ((\text{Component}_{\text{int}} - \text{Component}_{\text{Fc}}) / \text{Component}_{\text{int}}) * 100$, where $\text{Component}_{\text{int}}, \text{ kg/d}$

or g/d = the amount of intake of the component, and $\text{Component}_{\text{Fc}}$, kg/d or g/d = the amount of the component found in feces. Urinary volume was determined based on Tebbe and Weiss (2018) equation ($\text{Urinary volume, L/d} = 29 * \text{BW, kg} / \text{creatinine, mg/L}$) with coefficient adapted for heifers based on Chizzotti et al. (2008). Nitrogen balance (N_{Bal}) was determined by subtracting the N excretion (N_{Fc} and N_{Urn} for N found in feces and N found in urine, respectively) from N intake (N_{Int}): $N_{\text{bal}} = N_{\text{Int}} - N_{\text{Fc}} - N_{\text{Urn}}$.

3.3.5. Statistical analysis

The 2 trials were analyzed and reported separately. All statistical analysis was performed in JMP (v.17.2.0, SAS Institute Inc, Cary, NC) using the mixed effects model. The mixed model included the period and treatment as fixed effects, while heifers were used as the random effect. The least square means and standard errors were determined using effect leverage (REML method). Statistical significance was declared at $P\text{-value} \leq 0.05$ and tendency at $0.05 < P\text{-value} \leq 0.10$.

3.4. Results

3.4.1. Nutrient intake, fecal flows and apparent digestibility

Nutrient intake, fecal flows and digestibility of nutritional components is presented in Table 3.4 and 3.5 for Trial 1 and Trial 2, respectively. Intakes of DM, aNDFom and starch did not differ between two treatments in Trial 1. However, N intake was greater for HCP heifers compared with LCP heifers at 164.87 and 130.40 g/d, respectively ($P < 0.01$). Fecal flows of DM, N, aNDFom and starch were not affected by treatments. Digestibility of N and starch did not differ between treatments, but DM digestibility was greater for heifers fed the HCP diet at 65.57 % compared with 60.22 % for LCP heifers ($P < 0.01$).

Moreover, the aNDFom digestibility was higher for HCP treatment at 53.62 % compared with 48.78 % for LCP treatment ($P < 0.01$).

In Trial 2, intakes of DM and N were not affected by dietary treatments. Starch intake was greater for the HRDP group (1.88 and 1.51 kg/d for HRDP and LRDP treatment, respectively, $P < 0.01$), while there was a tendency ($P = 0.07$) for heifers fed LRDP diet to consume more aNDFom. Fecal flows of all components did not differ between treatments. Digestibility of DM was affected by treatments, with heifers fed the HRDP diet exhibiting lower digestibility at 66.35 % compared with 68.14 % of heifers fed the LRDP diet ($P = 0.05$). Similarly, aNDFom digestibility was decreased for HRDP treatment at 56.41 % compared with 61.58 % for LRDP treatment ($P < 0.01$). No difference was observed in N and starch digestibility between treatments.

3.4.2. Nitrogen allocation

Results on N allocation are shown in Table 3.6 and 3.7 for Trial 1 and Trial 2, respectively. In Trial 1, urinary volume was not affected by treatments and no difference between treatments was observed in N excreted in feces. In contrast, N excreted in urine was lower for LCP heifers compared with HCP ones (43.13 and 60.83 g/d for LCP and HCP, respectively; $P < 0.01$), resulting in 29.1 % less N excreted in the environment. Heifers under HCP treatment retained more N than heifers under LCP treatment (48.26 and 34.80 g/d for HCP and LCP, respectively; $P = 0.03$). As it is shown in Table 3.6, the percent of N intake that was excreted with urine was not affected by treatments. The percent of N intake that was excreted with feces was considerably higher for LCP treatment (33.44 % and 40.49 % for HCP and LCP treatment, respectively; $P < 0.01$), even though excreted N with feces as g/d was not different between

treatments. The retention of N expressed as a percentage of N intake was only numerically higher, at 28.64 % for HCP heifers compared with 25.86 % for LCP heifers.

In Trial 2, no difference was observed on urinary volume output. The allocation of N in intake, feces and urine was not affected by RDP level, with heifers under the HRDP treatment presenting only numerically higher N intake and N excretion. Moreover, N balance was not different between treatments. Similarly, N excreted in urine and feces and N retained expressed as percentage of N intake were not influenced by treatment.

3.4.3. Rumen and blood parameters

The effects of different CP and RDP levels on rumen and blood parameters are presented in Table 3.8 and 3.9 for Trial 1 and Trial 2, respectively. In Trial 1, higher dietary CP level increased the $\text{NH}_3\text{-N}$ concentration in the rumen, with HCP heifers exhibiting elevated concentration of 12.6 mg/dL compared with LCP heifers at 8.1 mg/dL ($P = 0.03$). It is noted that lower rumen pH for LCP heifers was observed at 6.8 compared with 7.0 for HCP heifers ($P = 0.03$). Blood urea level was greater for heifers provided the HCP diet compared with LCP heifers (4.8 and 3.3 mmol/L for HCP and LCP, respectively; $P < 0.01$). No effect of dietary treatments was observed on concentrations of albumin, creatinine, BHBA and NEFA in blood.

In Trial 2, higher proportion of RDP level resulted in a significant increase of rumen $\text{NH}_3\text{-N}$ concentration, with heifers receiving the HRDP diet responding with greater concentration of 10.9 mg/dL compared to 4.5 mg/dL of LRDP heifers ($P < 0.01$). The higher RDP levels did affect urea level, with heifers

under HRDP treatment presenting increased levels of 5.4 mmol/L compared with 3.7 mmol/L for heifers under LRDP diet ($P < 0.01$). The treatments did not alter rumen pH, while no difference was observed on albumin, creatinine, BHBA and NEFA levels in blood.

3.5. Discussion

3.5.1. Trial 1

The objective of Trial 1 was to examine the N utilization efficiency in response to 2 levels of dietary CP (HCP, LCP). To this end, in Trial 1 the formulated diets maintained the same level of forages and differed mostly in the inclusion of the soybean meal (9.3 versus 1.3 % DM for HCP and LCP, respectively; Table 3.1) and wheat bran (1.3 and 7.9 % DM for HCP and LCP, respectively; Table 3.1).

The DMI was similar or slightly lower with previous studies published evaluating N excretion of heifers of similar BW and age (Zanton and Heinrichs, 2009; Johansen et al., 2022). Nevertheless, DMI was not different between the treatments, although the aNDFom content of the treatment diets differed slightly (45.0 and 47.3 % DM, for HCP and LCP, respectively; Table 3.1). Although in other studies an increase of DMI has been reported with increased levels of CP in dairy heifers (Hoffman et al., 2001; Johansen et al., 2022), in our study this was not observed. However, this effect appears to reach a plateau, with increased CP level not affecting DMI above a threshold (Yang et al., 2022), while other studies did not report any effect of CP level on DMI (Broderick et al., 2015). In our study, the lack of CP effect on DMI may indicate that the CP levels of dietary treatments were in both cases adequate, while growth evaluation could have provided an insight into it; however, it was beyond the

scope of the current study. Additionally, DM digestibility was higher for HCP heifers compared with LCP heifers, in a manner consistent with aNDFom digestibility. Even though aNDFom intake was not different between treatments, the apparent digestibility of aNDFom was greater for heifers fed the HCP diet in Trial 1 (53.6 versus 48.8 % for HCP and LCP, respectively; $P < 0.01$; Table 3.4). The two dietary treatments fed to heifers had same forage inclusion, but different concentrate inclusion in the final TMR. The TMR of HCP treatment contained 1.3 % and 9.3 % DM wheat bran and soybean meal, respectively, while in contrast LCP treatment had 7.9 % and 1.3 % DM wheat bran and soybean meal (Table 3.1). As shown in Table 3.3, soybean meal had lower aNDFom concentration at 10.48 % DM than wheat bran at 42.94 % DM. Furthermore, based on CNPCS v6.5, considering a single potential digestible portion of NDF (pdNDF) pool, the degradation rate (k_d) of soybean meal is higher than wheat bran (6 and 5 % k_d , respectively) which may have led to increased aNDFom digestibility of HCP diet in combination with the lower aNDFom diet content (Van Amburgh et al., 2003; Raffrenato and Van Amburgh, 2010). Except for solid evidence as the diet composition, the different aNDFom composition is also shown in the ruminal pH difference (7.0 and 6.8 for HCP and LCP; P -value = 0.03; Table 3.8). This indicates that even though fermentation processes were not impaired, different fermentation products might have occurred between the two treatments, while any negative effects of surplus ruminal NH_3 is magnified at $\text{pH} > 7$ (Abdoun et al., 2006). Hence, the differences in DM and aNDFom digestibility are likely due to proportions of aNDFom coming from different feed ingredients and different degradability rates.

Moreover, there is evidence that higher N intake improves DM digestibility (Hoffman 2001, Marini and Van Amburgh 2005). Mlay et al. (2003) investigated the effect of N level on fiber digestibility using fistulated heifers and found that higher N levels improved DM digestibility, implying that increased N substrates improved rumen microbial activity. Structural carbohydrate bacteria (Bact_{SC}) require NH₃ to meet N requirements for growth, while when NH₃-N levels are reduced, Bact_{SC} cannot use fiber efficiently (Schwab and Broderick, 2017). In continuous culture fermenters, it has been suggested that at least 5 mg/dL of NH₃ should be present in rumen for efficient ruminal fermentation, while the minimum threshold without a safety cushion is considered the 2 mg/dL (Satter and Slyter, 1974). Therefore, the provision of lower N substrates for microbial growth, may lead to depression of aNDFom digestibility (Marini and Van Amburgh, 2003). Marini and Van Amburgh (2003) indicated a decrease in rumen NH₃-N concentrations from 10 mg/dL to 0.17 mg/dL, when CP levels were reduced from 15.6 % to 9.0 % DM, while NDF digestibility was decreased from 52.7 % to 39.9 %. Even though in our study rumen NH₃-N concentration in both treatments was well above minimum threshold (8.1 compared with 12.6 mg/dL for LCP and HCP, respectively; Table 3.8), it is more likely that aNDFom digestibility was mainly affected by the diet composition.

Heifers fed the HCP diet had greater N intake than heifers fed the LCP diet, whereas N digestibility did not differ between treatments. In contrast, Marini and Van Amburgh (2005), reported increased N digestibility as dietary N increased. The profound effects of adequate energy supplementation on N utilization by ruminal microbial population have been clearly demonstrated (Firkins, 1996). The maximization of microbial growth requires the synchronous

release of energy and protein, taking into consideration protein and carbohydrate sources' degradability. For example, rapidly available energy and protein consist of sugars and soluble protein or NPN, respectively, while slower available energy and protein, consist of NDF and N bound to NDF (NDIP). In our study, diets were isoenergetic (Table 3.1). Thus, N utilization appears not to have been limited by energy availability. Therefore, it can be presumed that no difference was observed on N digestibility, as maybe the LCP treatment was already adequate providing N substrates for microbial growth.

The higher level of dietary N increased the excretion of urinary N, which is in accordance with what other studies have reported (Marini and Van Amburgh, 2003; Zanton and Heinrichs, 2009; Johansen et al., 2022). This surplus of N corresponds well with blood urea levels, as LCP heifers exhibited moderate urea levels (3.3 mmol/L), while in the case of HCP heifers blood urea levels were considerably higher (4.8 mmol/L; Table 3.8). Similarly, several studies have reported increased levels of plasma urea N with increasing levels of dietary N (Marini and Van Amburgh, 2005; Zhang et al., 2017, Abeni et al., 2019). According to Brickell et al. (2009), moderate blood urea levels from 3.4 to 3.6 mmol/L for heifers from 6 to 15 months are considered optimal. Higher blood urea values indicate increased protein intake or protein catabolism due to energy depletion (Brickell et al., 2009). As mentioned before, treatments in our study were isoenergetic, therefore the difference between the treatments can be attributed to the different by design N intakes.

Furthermore, there was no difference in N excreted with feces between the treatments, indicating that with increased N intake, the excretion of surplus N was mainly through urine rather than feces (Van Amburgh et al., 2015). Fecal

N consists of several components, such as endogenous N (indigestible microbial N, microbial population originating in the rumen, intestine and hindgut) and undigested feed protein (Mason and Frederiksen, 1979; Lapierre et al., 2020). In general, non dietary fecal N is considered constant and not influenced by the dietary N intake (Zanton and Heinrichs, 2008). However, as N intake increases total fecal N may increase due to N content of undigested feeds. Zanton and Heinrichs (2008) conducting a meta-analysis observed that in weaned heifers (BW range from 56-472 kg), 82 and 95 % of total fecal N at the highest and lowest N intake, respectively, was non dietary fecal N. Similar to our findings, Marini and Van Amburgh (2005) reported no effect of CP level on fecal N excretion, even though N excreted with feces increased only arithmetically at higher levels of CP. Heifers under the HCP treatment exhibited increased N retention compared with LCP treatment (48.3 versus 34.8 g/d for HCP and LCP, respectively, P -value = 0.03; Table 3.6). Similarly, Zanton and Heinrichs (2009), demonstrated that there is a linear increase in N retention with increasing levels of N intake. Moreover, based on data from Marini and Van Amburgh (2005), it was calculated that N retention increased from 19.6 to 32.4 g/d, when CP level increased from 9.0 to 18.6 % DM, while Zhang et al. (2017) report no effect of three CP levels; 10.2, 11.9 and 13.5 % DM, on N retention. However, increased N retention does not necessarily mean increased NUE. Indeed, based on data from Marini and Van Amburgh (2005), an NUE of 22.4 and 18.1 % was calculated for the CP levels of 9.0 and 18.6 % DM, respectively. In our study, no difference was observed between the treatments on N balance when expressed as a percentage of N intake. Even though, N balance appears to be higher for HCP treatment, growth has not

been evaluated; yet, similar NUE between treatments, points out that a lower CP level might be beneficial when the effort focuses on minimizing excreted N in the environment.

3.5.2. Trial 2

The objective of Trial 2 was to examine the N utilization efficiency in response to 2 levels of RDP (HRDP, LRDP) in a LCP diet. In Trial 2, similarly with Trial 1, the same level of forages was maintained, but alterations in concentrate levels and sources were made to maintain the same CP level and increase the RDP level. In brief, LRDP diets contained soybean meal (inclusion at 5.1 and 3.2 % DM, respectively; Table 3.2) without the addition of urea and wheat bran, while HRDP contained soybean meal, wheat bran and urea (inclusion at 3.6, 2.6 and 0.7 % DM, respectively; Table 3.2).

Even though DM intake did not differ between treatments, DM digestibility was higher for LRDP heifers. This corresponds also to albumin levels, as heifers under LRDP treatment had albumin levels at 32.1 g/L compared with HRDP heifers at 31.3 g/L (Table 3.9). Serum albumin is a carrier protein (Jahanban-Esfahlan et al., 2019), with higher levels being related to nutrient absorption (Machado et al., 2020). This means that when higher levels of available nutrients are to be absorbed, higher albumin levels are required so as to be utilized for different compound transfer (e.g. fatty acids, amino acids; (Machado et al., 2020). Moreover, in Trial 2, heifers under the LRDP treatment exhibited a tendency for increased aNDFom intake (4.4 versus 4.7 kg/d for HRDP and LRDP, respectively; $P = 0.07$; Table 3.5), which led to higher aNDFom digestibility than heifers under HRDP treatment (56.41 versus 61.58 % for HRDP and LRDP, respectively; $P < 0.01$; Table 3.5). Corea et al. (2020)

reported a tendency for higher apparent NDF digestibility of heifers fed a level of RDP at 64 % CP compared with 74 % CP, which was attributed to different NDF proportions. In our study, even though treatment diets have similar levels of aNDFom, there were alterations regarding NDF sources. As shown in Table 3.2, HRDP diets contained soybean meal and wheat bran, while LRDP mainly soybean meal and soypass. As in Trial 1, herein different NDF proportions and degradation rates may have improved DM and aNDFom digestibility for LRDP heifers.

We observed no difference on N intake and N digestibility, when RDP level was altered. Similarly, previous studies that tested differing levels of RDP at the same CP level did not report any effects of RDP on nutrient intake and digestibility (Gabler and Heinrichs, 2003; Silva et al., 2018). It is expected that not only the CP level, but also the RDP may be limiting at low levels of supply, compromising microbial growth and nutrient degradation (Corea et al., 2020). As a consequence of limited N substrates, N is recycled back to the rumen from the liver in the form of urea in order to maintain a positive N balance in the ruminal environment (Batista et al., 2017). For LRDP heifers, rumen $\text{NH}_3\text{-N}$ was decreased at 4.5 compared with 10.9 mg/dL for HRDP heifers (P -value < 0.01; Table 3.9), which is in accordance with the fact that lower rumen NH_3 levels are expected when RDP levels are reduced (Reynal and Broderick, 2005). In both treatments, rumen $\text{NH}_3\text{-N}$ concentration was well above minimum threshold (2 mg/L), while rumen $\text{NH}_3\text{-N}$ in LRDP heifers was slightly lower than suggested level (4.5 compared with 5 mg/L; Satter and Slyter, 1974)). However, it is noted that rumen $\text{NH}_3\text{-N}$ concentration is not constant, presenting fluctuations and a reduction during the day and until next feeding (Zanton et al., 2007). Therefore,

taking into consideration that DM and aNDFom digestibility were higher for LRDP heifers, there is an indication that a greater portion of N was recycled towards microbial protein synthesis rather than being excreted with urine.

The higher $\text{NH}_3\text{-N}$ in heifers fed the HRDP diet resulted in corresponding effects on urea in blood (5.4 mmol/L compared with 3.7 mmol/L for HRDP and LRDP heifers, respectively, P -value < 0.01; Table 3.9), meaning that the absorption of rumen $\text{NH}_3\text{-N}$ was greater for HRDP heifers, with higher detoxification in the liver and formation of urea, which is the end product of N metabolism (Hailemariam et al., 2021). As a consequence, we detected a tendency for an increase in N excreted with urine, indicating that microbial population reached their potential to incorporate NH_3 into microbial protein, leading to inefficient use of N. This probably led to a tendency for decreased NUE for HRDP heifers (17.4 and 23 % for HRDP and LRDP, respectively, P -value = 0.1; Table 3.7). Nevertheless, the animal number and inter-animal variations may be responsible for causing no significant differences. Accordingly, N balance and fecal N excretion, although arithmetically higher and lower, respectively for LRDP heifers, were not different. Similar to our findings, Corea et al. (2020) under tropical conditions observed no effect of different RDP levels (RDP at 64.0% and 76.0 % CP), respectively on N excretion and retention. Silva et al. (2018) examined the effects of RDP and RUP levels on intakes, digestibility and N utilization. Diets contained levels of RDP at 61.1, 55.9, 51.1 and 46.0 % CP, while CP levels were similar among treatments at approximately 15.2 % DM. In contrast with our results, it was found that N intake and N fecal excretion were affected by RDP level, while a tendency for reduction of urinary N was observed with decreased levels of RDP

(97.3, 97.4, 89.7 and 85.5 g/d, for RDP at 61.1, 55.9, 51.1 and 46.0 % CP, respectively). Moreover, a tendency for increased N retention was observed at the lower levels of RDP and thus higher levels of RUP (Silva et al., 2018).

All things considered, in a constant level of CP we did not observe great differences on N allocation and excretion. The fact that the considerably lower concentration of rumen available N appeared not to be limiting for LRDP heifers, might indicate that heifers physiologically adapted with mechanisms such as N recycling to avoid negative fermentation implications, thus exhibiting improved nutrient utilization. Compared with the previously published research results, for example Silva et al. (2018), in our study the CP level was set lower and RDP level was set higher. Thus, it is possible that differences in RDP treatments of our study were not large enough to detect any significant effect on N excretion. Nevertheless, even under these conditions, we observed a tendency for decreased NUE in heifers fed the HRDP diet. Moreover, when treatment diets were formulated using the CNCPS (version 6.5), based on model predictions rumen NH_3 requirements were estimated at 111.0 % for LRDP heifers. Rumen NH_3 balance is recommended to range between 110 to 120 % of requirements (Foskolos and Plomaritou, 2023). Thus, maybe this is the reason why ruminal activity was not impaired, although rumen NH_3 -N and plasma urea in heifers under LRDP treatment were lower compared with HRDP treatment.

3.6. Conclusions

The higher CP level affected positively N balance, however in the expense of higher N concentration in rumen and urine, leading to higher N loss in the environment. Nonetheless, by feeding the same CP level and a lower

RDP level we observed a tendency for increased NUE and reduced N excretion via urine.

3.7. References

- Abdoun, K., Stumpff, F., Martens, H., 2006. Ammonia and urea transport across the rumen epithelium: A review. *Animal Health Research Reviews* 7, 43-59. doi: <https://doi.org/10.1017/S1466252307001156>.
- Barros, T., Reed, K.F., Olmos Colmenero, J.J., Wattiaux, M.A., 2019. Short communication: Milk urea nitrogen as a predictor of urinary nitrogen and urea nitrogen excretions of late-lactation dairy cows fed nitrogen-limiting diets. *Journal of Dairy Science* 102, 1601-1607. doi: <https://doi.org/10.3168/jds.2018-14551>.
- Batista, E.D., Detmann, E., Valadares Filho, S.C., Titgemeyer, E.C., Valadares, R.F.D., 2017. The effect of cp concentration in the diet on urea kinetics and microbial usage of recycled urea in cattle: A meta-analysis. *Animal* 11, 1303-1311. doi: <https://doi.org/10.1017/S1751731116002822>.
- Brickell, J.S., McGowan, M.M., Wathes, D.C., 2009. Effect of management factors and blood metabolites during the rearing period on growth in dairy heifers on UK farms. *Domestic Animal Endocrinology* 36, 67-81. doi: <https://doi.org/10.1016/j.domaniend.2008.10.005>.
- Broderick, G.A., Faciola, A.P., Armentano, L.E., 2015. Replacing dietary soybean meal with canola meal improves production and efficiency of lactating dairy cows. *Journal of Dairy Science* 98, 5672-5687. doi: <https://doi.org/10.3168/jds.2015-9563>.
- Bussink, D.W., Oenema, O., 1998. Ammonia volatilization from dairy farming systems in temperate areas: A review. *Nutrient Cycling in Agroecosystems* 51, 19-33. doi: <https://doi.org/10.1023/A:1009747109538>.

- Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., van Vuuren, A.M., 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184-1196. doi: <https://doi.org/10.1017/S1751731110000911>.
- Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and ammonia. *Clinical Chemistry* 8, 130-132. doi: <https://doi.org/10.1093/clinchem/8.2.130>.
- Chizzotti, M.L., Valadares Filho, S.d.C., Valadares, R.F.D., Chizzotti, F.H.M., Tedeschi, L.O., 2008. Determination of creatinine excretion and evaluation of spot urine sampling in holstein cattle. *Livestock Science* 113, 218-225. doi: <https://doi.org/10.1016/j.livsci.2007.03.013>.
- Corea, E.E., Castro-Montoya, J., Mendoza, M.V., López, F.M., Martinez, A., Alvarado, M.E., Moreno, C., Broderick, G.A., Dickhoefer, U., 2020. Effect of forage source and dietary rumen-undegradable protein on nutrient use and growth in dairy heifers. *Animal Feed Science and Technology* 269, 114658. doi: <https://doi.org/10.1016/j.anifeedsci.2020.114658>.
- Davidson, S., Hopkins, B.A., Diaz, D.E., Bolt, S.M., Brownie, C., Fellner, V., Whitlow, L.W., 2003. Effects of amounts and degradability of dietary protein on lactation, nitrogen utilization, and excretion in early lactation holstein cows. *Journal of Dairy Science* 86, 1681-1689. doi: [https://doi.org/10.3168/jds.S0022-0302\(03\)73754-0](https://doi.org/10.3168/jds.S0022-0302(03)73754-0).
- Dijkstra, J., Oenema, O., van Groenigen, J.W., Spek, J.W., van Vuuren, A.M., Bannink, A., 2013. Diet effects on urine composition of cattle and N₂O emissions. *Animal* 7, 292-302. doi: <https://doi.org/10.1017/S1751731113000578>.

- Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T., Webster, G., 1989. A body condition scoring chart for holstein dairy cows. *Journal of Dairy Science* 72, 68-78. doi: [https://doi.org/10.3168/jds.S0022-0302\(89\)79081-0](https://doi.org/10.3168/jds.S0022-0302(89)79081-0).
- Erisman, J.W., Leach, A., Bleeker, A., Atwell, B., Cattaneo, L., Galloway, J., 2018. An integrated approach to a nitrogen use efficiency (NUE) indicator for the food production–consumption chain. *Sustainability* 10, 925. doi: <https://doi.org/10.3390/su10040925>.
- Firkins, J.L., 1996. Maximizing microbial protein synthesis in the rumen. *The Journal of Nutrition* 126, 1347S-1354S. doi: https://doi.org/10.1093/jn/126.suppl_4.1347S.
- Foskolos, A., Plomaritou, A., 2023. Guest editorial: Holistic nutritional management as a tool to mitigate nitrogen pollution from dairy cattle farming. *The Journal of Agricultural Science*, 1-17. doi: <https://doi.org/10.1017/S0021859623000254>.
- Gabler, M.T., Heinrichs, A.J., 2003. Altering soluble and potentially rumen degradable protein for prepubertal Holstein heifers. *Journal of Dairy Science* 86, 2122-2130. doi: [https://doi.org/10.3168/jds.S0022-0302\(03\)73802-8](https://doi.org/10.3168/jds.S0022-0302(03)73802-8).
- Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., Cosby, B.J., 2003. The nitrogen cascade. *BioScience* 53, 341-356. doi: [https://doi.org/10.1641/0006-3568\(2003\)053\[0341:Tnc\]2.0.Co;2](https://doi.org/10.1641/0006-3568(2003)053[0341:Tnc]2.0.Co;2).
- Guo, Y.Q., Tong, B.X., Wu, Z.G., Ma, W.Q., Ma, L., 2019. Dietary manipulation to reduce nitrogen and phosphorus excretion by dairy cows. *Livestock Science* 228, 61-66. doi: <https://doi.org/10.1016/j.livsci.2019.08.001>.

- Hailemariam, S., Zhao, S., He, Y., Wang, J., 2021. Urea transport and hydrolysis in the rumen: A review. *Animal Nutrition* 7, 989-996. doi: <https://doi.org/10.1016/j.aninu.2021.07.002>.
- Heinrichs, A.J., Rogers, G.W., Cooper, J.B., 1992. Predicting body weight and wither height in Holstein heifers using body measurements. *Journal of Dairy Science* 75, 3576-3581. doi: [https://doi.org/10.3168/jds.S0022-0302\(92\)78134-X](https://doi.org/10.3168/jds.S0022-0302(92)78134-X).
- Hoffman, P.C., Esser, N.M., Bauman, L.M., Denzine, S.L., Engstrom, M., Chester-Jones, H., 2001. Short communication: Effect of dietary protein on growth and nitrogen balance of Holstein heifers. *Journal of Dairy Science* 84, 843-847. doi: [https://doi.org/10.3168/jds.S0022-0302\(01\)74542-0](https://doi.org/10.3168/jds.S0022-0302(01)74542-0).
- Hristov, A.N., Hanigan, M., Cole, A., Todd, R., McAllister, T.A., Ndegwa, P.M., Rotz, A., 2011. Review: Ammonia emissions from dairy farms and beef feedlots. *Canadian Journal of Animal Science* 91, 1-35. doi: <https://doi.org/10.4141/cjas10034>.
- Huhtanen, P., Ahvenjärvi, S., 2022. Review: Problems in determining metabolisable protein value of dairy cow diets and the impact on protein feeding. *Animal* 16, 100539. doi: <https://doi.org/10.1016/j.animal.2022.100539>.
- Jahanban-Esfahlan, A., Ostadrahimi, A., Jahanban-Esfahlan, R., Roufegarinejad, L., Tabibiazar, M., Amarowicz, R., 2019. Recent developments in the detection of bovine serum albumin. *International Journal of Biological Macromolecules* 138, 602-617. doi: <https://doi.org/10.1016/j.ijbiomac.2019.07.096>.

- Johansen, M., Madsen, P.A., Brask-Pedersen, D.N., Lund, P., 2022. Effect of age and dietary crude protein level on nitrogen excretion in dairy heifers. *Livestock Science* 264, 105058. doi: <https://doi.org/10.1016/j.livsci.2022.105058>.
- Lapierre, H., Martineau, R., Hanigan, M.D., van Lingen, H.J., Kebreab, E., Spek, J.W., Ouellet, D.R., 2020. Review: Impact of protein and energy supply on the fate of amino acids from absorption to milk protein in dairy cows. *Animal* 14, s87-s102. doi: <https://doi.org/10.1017/S1751731119003173>.
- Machado, A.F., Guimarães, S.E.F., Guimarães, J.D., Santos, G.M., Silva, A.L., Silva, Y.F.R.S., Lollobrigida Netto, D.S., Correa, P.V.F., Marcondes, M.I., 2020. Effect of protein supplement level on the productive and reproductive parameters of replacement heifers managed in intensive grazing systems. *Plo One* 15, e0239786. doi: <https://doi.org/10.1371/journal.pone.0239786>.
- Marini, J.C., Van Amburgh, M.E., 2003. Nitrogen metabolism and recycling in holstein heifers. *Journal of Animal Science* 81, 545-552. doi: <https://doi.org/10.2527/2003.812545x>.
- Marini, J.C., Van Amburgh, M.E., 2005. Partition of nitrogen excretion in urine and the feces of holstein replacement heifers. *Journal of Dairy Science* 88, 1778-1784. doi: [https://doi.org/10.3168/jds.S0022-0302\(05\)72852-6](https://doi.org/10.3168/jds.S0022-0302(05)72852-6).
- Mason, V.C., Frederiksen, J.H., 1979. Partition of the nitrogen in sheep faeces with detergent solutions, and its application to the estimation of the true digestibility of dietary nitrogen and the excretion of non dietary faecal

- nitrogen. Zeitschrift für Tierphysiologie Tierernährung und Futtermittelkunde 41, 121-131. doi: <https://doi.org/10.1111/j.1439-0396.1978.tb00573.x>.
- Mlay, P.S., Pereka, A.E., Weisbjerg, M.R., Hvelplund, T., Madsen, J., 2003. Digestion and passage kinetics of fibre in mature dairy heifers maintained on poor quality hay as affected by the source and level of nitrogen supplementation. Animal Feed Science and Technology 109, 19-33. doi: [https://doi.org/10.1016/S0377-8401\(03\)00211-6](https://doi.org/10.1016/S0377-8401(03)00211-6).
- NASEM, 2021. Nutrient requirements of dairy cattle, 8th revised edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/25806>.
- Raffrenato, E., Ross, D.A., Van Amburgh, M.E., 2018. Development of an in vitro method to determine rumen undigested and form for use in feed evaluation. Journal of Dairy Science 101, 9888-9900. doi: <https://doi.org/10.3168/jds.2018-15101>.
- Reynal, S.M., Broderick, G.A., 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. Journal of Dairy Science 88, 4045-4064. doi: [https://doi.org/10.3168/jds.S0022-0302\(05\)73090-3](https://doi.org/10.3168/jds.S0022-0302(05)73090-3).
- Satter, L.D., Slyter, L.L., 1974. Effect of ammonia concentration of rumen microbial protein production in vitro. British Journal of Nutrition 32, 199-208. doi: <https://doi.org/10.1079/bjn19740073>.
- Schwab, C.G., Broderick, G.A., 2017. A 100-year review: Protein and amino acid nutrition in dairy cows. Journal of Dairy Science 100, 10094-10112. doi: <https://doi.org/10.3168/jds.2017-13320>.

- Silva, A.L., Detmann, E., Rennó, L.N., Pedroso, A.M., Fontes, M.M.S., Morais, V.C., Sguizzato, A.L.L., Abreu, M.B., Rotta, P.P., Marcondes, M.I., 2018. Effects of rumen undegradable protein on intake, digestibility and rumen kinetics and fermentation characteristics of dairy heifers. *Animal Feed Science and Technology* 244, 1-10. doi: <https://doi.org/10.1016/j.anifeedsci.2018.07.019>.
- Sutton, M.A., Howard, C.M., Kanter, D.R., Lassaletta, L., Móríng, A., Raghuram, N., Read, N., 2021. The nitrogen decade: Mobilizing global action on nitrogen to 2030 and beyond. *One Earth* 4, 10-14. doi: <https://doi.org/10.1016/j.oneear.2020.12.016>.
- Sutton, M.A., van Grinsven, H., Billen, G., Bleeker, A., Bouwman, A.F., Bull, K., Erisman, J.W., Grennfelt, P., Grizzetti, B., Howard, C.M., Oenema, O., Spranger, T., Winiwarter, W., 2011. Summary for policy makers. In *The european nitrogen assessment: Sources, effects and policy perspectives* (ed. Bleeker, A., Grizzetti, B., Howard, C.M., Billen, G., van Grinsven, H., Erisman, J.W., Sutton, M.A. and Grennfelt, P.), Cambridge University Press, Cambridge, UK, p. xxiv-xxxiv. doi: <https://doi.org/10.1017/CBO9780511976988.002>.
- Tebbe, A.W., Weiss, W.P., 2018. Evaluation of creatinine as a urine marker and factors affecting urinary excretion of magnesium by dairy cows. *Journal of Dairy Science* 101, 5020-5032. doi: <https://doi.org/10.3168/jds.2017-14098>.
- Tedeschi, L.O., Fox, D.G., Fonseca, M.A., Cavalcanti, L.F.L., 2015. Models of protein and amino acid requirements for cattle. *Revista Brasileira de*

- Zootecnia 44, 109-132. doi: <https://doi.org/10.1590/S1806-92902015000300005>.
- Van Amburgh, M.E., Collao-Saenz, E.A., Higgs, R.J., Ross, D.A., Recktenwald, E.B., Raffrenato, E., Chase, L.E., Overton, T.R., Mills, J.K., Foskolos, A., 2015. The cornell net carbohydrate and protein system: Updates to the model and evaluation of version 6.5. *Journal of Dairy Science* 98, 6361-6380. doi: <https://doi.org/10.3168/jds.2015-9378>.
- Van Amburgh, M.E., Soberon, F., Meyer, M.J., Molano, R.A., 2019. Symposium review: Integration of postweaning nutrient requirements and supply with composition of growth and mammary development in modern dairy heifers. *Journal of Dairy Science* 102, 3692-3705. doi: <https://doi.org/10.3168/jds.2018-15270>.
- Yang, C.T., Ferris, C.P., Yan, T., 2022. Effects of dietary crude protein concentration on animal performance and nitrogen utilisation efficiency at different stages of lactation in holstein-friesian dairy cows. *Animal* 16, 100562. doi: <https://doi.org/10.1016/j.animal.2022.100562>.
- Zanton, G.I., Gabler, M.T., Heinrichs, A.J., 2007. Manipulation of soluble and rumen-undegradable protein in diets fed to postpubertal dairy heifers. *Journal of Dairy Science* 90, 978-986. doi: [https://doi.org/10.3168/jds.S0022-0302\(07\)71582-5](https://doi.org/10.3168/jds.S0022-0302(07)71582-5).
- Zanton, G.I., Heinrichs, A.J., 2008. Analysis of nitrogen utilization and excretion in growing dairy cattle. *Journal of Dairy Science* 91, 1519-1533. doi: <https://doi.org/10.3168/jds.2007-0624>.
- Zanton, G.I., Heinrichs, A.J., 2009. Digestion and nitrogen utilization in dairy heifers limit-fed a low or high forage ration at four levels of nitrogen

intake1. Journal of Dairy Science 92, 2078-2094. doi:
<https://doi.org/10.3168/jds.2008-1712>.

Zhang, B., Wang, C., Liu, H., Liu, J., Liu, H., 2017. Effects of dietary protein level on growth performance and nitrogen excretion of dairy heifers. Asian-Australasian Journal of Animal Sciences 30, 386-391. doi:
<https://doi.org/10.5713/ajas.16.0214>.

3.8. Tables and Figures

Table 3.1. Ingredient inclusion (%DM), diet composition and evaluation of treatment diets containing low and high crude protein levels (CP; LCP, HCP) in Trial 1.

	HCP ¹	LCP ²
Ingredient, % DM		
Corn silage	40.9	40.9
Wheat straw	13.0	13.0
Alfalfa hay	25.4	25.3
Corn grain	9.0	10.4
Soybean meal	9.3	1.3
Wheat bran	1.3	7.9
Urea	0.6	0.6
Vitamin and mineral mix ³	0.5	0.6
Diet composition ⁴		
DM, %	54.9	54.8
CP, % DM	14.9	12.1
Starch, %	19.5	21.9
aNDFom, %DM	45.0	47.3
uNDFom, % DM	16.2 ± 3.9	16.8 ± 3.5
uNDFom, %aNDFom	34.6 ± 5.7	35.9 ± 5.6
Ash, %DM	5.7	5.6
Diet evaluation ⁵		
MP, %Rqd	115.7	103.6
ME, %Rqd	105.1	103.4

ME, Mcal/kg	2.3	2.3
Rumen _{NH3} , %Rqd	148.0	123.0

¹HCP = high crude protein level; ²LCP = low crude protein level.

³The mineral and vitamin mix contained: 23.8 % Ca; 3.0 % P; 2.0 % Mg; 7.6 % Na; 4000 mg/ kg of ZnO; 1600 mg/ kg of MnO; 2100 mg/kg of FeSO₄; 50 mg/kg of Ca(IO₃)₂; 25 mg/kg of CoCO₃ 5%; 9 mg/kg of Na₂SeO₃ 4.5 %; 270000 IU/kg of vitamin A; 40000 IU/kg of vitamin D₃; 200 mg/kg of vitamin E; 100 mg/kg of vitamin B₃; 1600 mg/kg of vitamin B₁₂; 16 mg/kg of vitamin B₁; 10000 mg/kg of saccharomyces cerevisae.

⁴DM = dry matter; OM = organic matter; CP = crude protein; aNDFom = neutral detergent fiber determined with the use of heat stable α -amylase and corrected for ash content; uNDFom = undigestible aNDFom.

⁵Estimated with the use of Cornell Net Carbohydrate System (CNCPS, v6.5); MP = metabolizable protein expressed as a percentage of requirements; ME = metabolizable energy expressed as a percentage of requirements; RumenNH₃ = Rumen ammonia expressed as a percentage of requirements.

Table 3.2. Ingredient inclusion (%DM), diet composition and evaluation of treatment diets containing high and low rumen degradable protein levels (HRDP and LRDP, respectively) in Trial 2.

	HRDP ¹	LRDP ²
Ingredient, DM %		
Corn silage	49.5	49.5
Wheat straw	11.0	11.0
Alfalfa hay	21.4	21.4
Corn grain	10.3	9.0
Soybean meal	3.6	3.2
Soy pass	-	5.1
Wheat bran	2.6	-
Urea	0.7	-
Limestone	0.5	0.4
Calcium monophosphate	0.2	0.2
Salt	0.1	0.1
Vitamin and mineral mix ³	0.1	0.1
Diet composition ⁴		
DM, %	49.8	49.8
CP, % DM	13.0	13.0
RDP, % CP	75.2	64.3
RUP, % CP	24.8	35.7
Starch, % DM	22.4	20.8
aNDFom, %DM	46.2	46.1
uNDFom, % DM	14.8 ± 2.5	15.0 ± 0.1

uNDFom, %aNDFom	29.7 ± 4.8	31.6 ± 2.0
Ash, %DM	6.2	6.3
Diet evaluation ⁵		
MP, %Rqd	108.9	132.6
ME, %Rqd	103.3	106.9
ME, Mcal/kg	2.3	2.3
Rumen _{NH3} , %Rqd	134.0	111.0

¹HRDP = high rumen degradable protein level; ²LRDP = low rumen degradable protein level.

³The mineral and vitamin mix contained: 9.6 % Ca; 0.05 % P; 0.01 % Mg; 0.01 % Na; 40000 mg/ kg of ZnO; 32500 mg/ kg of MnO; 40000 mg/kg of FeSO₄; 1250 mg/kg of Ca(IO₃)₂; 7500 mg/kg of CuSO₄; 125 mg/kg of Na₂SeO₃; 8000000 IU/kg of vitamin A; 800000 IU/kg of vitamin D₃; 50000 mg/kg of vitamin E; 750 mg/kg of vitamin K₃; 2000 mg/kg of vitamin B₁; 1500 mg/kg of vitamin B₂; 3500 mg/kg of vitamin B₅; 1750 mg/kg of vitamin B₆; 7500 mg/kg of vitamin B₁₂; 100000 mg/kg of vitamin C, 7500 mg/kg of niacin; 110 mg/kg of folic acid; 38 mg/k fog biotin; 125000 mg/kg of choline chloride.

⁴DM = dry matter; OM = organic matter; CP = crude protein; aNDFom = neutral detergent fiber determined with the use of heat stable α -amylase and corrected for ash content; uNDFom = undigestible aNDFom.

⁵Estimated with the use of Cornell Net Carbohydrate System (CNCPS, v6.5); MP = metabolizable protein expressed as a percentage of requirements; ME = metabolizable energy expressed as a percentage of requirements; RDP = rumen degradable protein; RUP = rumen undegradable protein; Rumen_{NH3} = Rumen ammonia expressed as a percentage of requirements.

Table 3.3. Chemical composition of feed ingredients used in diets of dairy replacement heifers for Trial 1 and Trial 2.

Item ¹	Corn silage	Wheat straw	Alfalfa hay	Corn	Soybean meal	Wheat bran	Soypass
DM, %	33.5 ± 1.3	91.0 ± 1.2	87.9 ± 2.3	85.4 ± 0.9	88.0 ± 1.1	87.3 ± 1.1	88.5
OM, %DM	95.8 ± 0.5	93.9 ± 0.8	92.2 ± 0.8	98.6 ± 0.4	93.0 ± 0.7	94.9 ± 0.5	93.0
CP, % DM	7.2 ± 0.5	3.4 ± 0.4	15.3 ± 2.8	8.3 ± 0.9	49.7 ± 3.0	17.5 ± 0.4	47.5
Starch, %	30.0 ± 2.5	1.7 ± 0.8	1.1 ± 0.6	70.3 ± 2.1	2.6 ± 1.9	23.1 ± 5.2	0.3
aNDFom, %DM	45.3 ± 6.1	80.9 ± 4.6	53.8 ± 4.7	13.2 ± 1.1	10.0 ± 2.4	42.8 ± 3.8	35.4
Ash, %DM	4.2 ± 0.5	6.1 ± 0.8	7.77 ± 0.8	1.4 ± 0.4	7.1 ± 0.7	5.1 ± 0.5	7.0

¹DM = dry matter; OM = organic matter; CP = crude protein; aNDFom = neutral detergent fiber determined with the use of heat stable α -amylase and corrected for ash content.

Table 3.4. Daily nutrient intakes, fecal flows and apparent digestibility of replacement heifers fed diets differing in crude protein (CP) level.

Item ¹	HCP ²	LCP ³	SEM	P-value
DM, kg/d				
Intake	7.2	7.0	0.3	0.41
Fecal flow	2.5	2.6	0.1	0.41
Digestibility, %	65.6	60.2	0.6	<0.01
N, g/d				
Intake	164.9	130.4	5.7	<0.01
Fecal flow	55.0	51.9	1.7	0.11
Digestibility, %	62.2	60.5	0.8	0.14
aNDFom, kg/d				
Intake	3.1	3.0	0.2	0.43
Fecal flow	1.5	1.6	0.1	0.13
Digestibility, %	53.6	48.8	0.7	<0.01
Starch, kg/d				
Intake	1.0	1.0	0.04	0.21
Fecal flow	0.02	0.08	0.03	0.09
Digestibility, %	94.8	96.0	0.5	0.11

¹DM = dry matter; N = nitrogen; aNDFom = neutral detergent fiber determined with the use of heat stable α -amylase and corrected for ash content.

²HCP = high crude protein level; ³LCP = low crude protein level.

Table 3.5. Daily nutrient intakes, fecal flows and apparent digestibility of replacement heifers fed diets differing in rumen degradable protein level (RDP).

Item ¹	HRDP ²	LRDP ³	SEM	P-value
DM, kg/d				
Intake	9.3	9.4	0.4	0.71
Fecal flow	3.1	3.0	0.2	0.50
Digestibility, %	66.4	68.1	0.6	0.05
N, g/d				
Intake	195.1	192.2	7.7	0.63
Fecal flow	74.3	73.2	3.5	0.76
Digestibility, %	61.3	62.2	0.7	0.41
aNDFom, kg/d				
Intake	4.4	4.7	0.2	0.07
Fecal flow	1.9	1.8	0.1	0.33
Digestibility, %	56.4	61.6	0.6	<0.01
Starch, kg/d				
Intake	1.9	1.5	0.1	<0.01
Fecal flow	0.03	0.03	0.01	0.34
Digestibility, %	98.3	98.3	0.2	0.91

¹DM = dry matter; N = nitrogen; aNDFom = neutral detergent fiber determined with the use of heat stable α -amylase and corrected for ash content.

²HRDP = high rumen degradable protein level; ³LRDP = low rumen degradable protein level.

Table 3.6. Urinary volume, nitrogen allocation and nitrogen balance of replacement heifers fed diets differing in crude protein (CP) level.

Item ¹	HCP ²	LCP ³	SEM	P-value
Urinary volume, L/d	6.9	6.4	0.6	0.37
Nitrogen balance				
N _{Int} , g/d	164.9	130.4	5.7	<0.01
N _{Um} , g/d	60.8	43.1	3.7	<0.01
N _{Fc} , g/d	55.0	51.9	1.7	0.11
N _{Bal} , g/d	48.3	34.8	6.4	0.03
N _{Um} : N _{Int} , %	37.9	33.7	3.0	0.11
N _{Fc} : N _{Int} , %	33.4	40.5	0.7	<0.01
N _{Bal} : N _{Int} , %	28.6	25.9	3.4	0.36

¹N_{Int} = N intake; N_{Fc} = N contained in feces; N_{Um} = N contained in urine; N_{Bal} = N balance.

²HCP = high crude protein level; ³LCP = low crude protein level.

Table 3.7. Urinary volume, nitrogen allocation and nitrogen balance of replacement heifers fed diets differing in levels of rumen degradable protein (RDP).

Item ¹	HRDP ²	LRDP ³	SEM	P-value
Urinary volume, L/d	8.9	9.0	1.1	0.92
Nitrogen balance				
N _{Int} , g/d	195.1	192.2	7.7	0.63
N _{Um} , g/d	85.8	75.1	6.6	0.10
N _{Fc} , g/d	74.3	73.2	3.5	0.76
N _{Bal}	35.0	44.0	5.1	0.19

N _{Um} : N _{Int} , %	43.9	39.2	2.9	0.14
N _{Fc} : N _{Int} , %	38.7	37.8	0.7	0.41
N _{Bal} : N _{Int} , %	17.4	23.0	2.7	0.10

¹N_{Int} = N intake; N_{Fc} = N contained in feces; N_{Um} = N contained in urine; N_{Bal} = N balance.

²HRDP = high rumen degradable protein level; ³LRDP = low rumen degradable protein level.

Table 3.8. Rumen and blood parameters of heifers offered diets with different level of crude protein (CP).

Item ¹	HCP ²	LCP ³	SEM	P-value
Rumen				
pH	7.0	6.8	0.1	0.03
NH ₃ -N, mg/dL	12.6	8.1	1.1	0.03
Blood				
Albumin, g/L	30.8	29.9	1.3	0.59
Creatinine, µmol/L	62.7	59.3	5.4	0.35
Urea, mmol/L	4.8	3.3	0.3	<0.01
BHBA, µmol/L	494.6	488.9	37.7	0.92
NEFA, µmol/L	342.4	322.2	11.0	0.31

¹NH₃-N = rumen ammonia N; BHBA = beta-hydroxy-butyric acid; NEFA = non-esterified fatty acids.

²HCP = high crude protein level; ³LCP = low crude protein level.

Table 3.9. Rumen and blood parameters of heifers offered different levels of rumen degradable protein (RDP).

Item ¹	HRDP ²	LRDP ³	SEM	P-value
Rumen				
pH	6.6	6.5	0.1	0.32
NH ₃ -N, mg/dL	10.9	4.5	0.9	<0.01
Blood				
Albumin, g/L	31.3	32.1	0.4	0.05
Creatinine, µmol/L	63.4	67.3	4.1	0.21
Urea, mmol/L	5.4	3.7	0.2	<0.01
BHBA, µmol/L	544.4	536.4	20.9	0.78
NEFA, µmol/L	210.0	206.7	10.1	0.81

¹NH₃-N = rumen ammonia N; BHBA = beta-hydroxy-butyric acid; NEFA = non-esterified fatty acids.

²HRDP = high rumen degradable protein level; ³LRDP = low rumen degradable protein level.

Chapter 4

Holistic nutritional management as a tool to mitigate

nitrogen pollution from dairy cattle farming

4.1. Introduction

For at least the last three decades, nitrogen (N) pollution from agricultural activities has been a central social and scientific issue. A series of environmental policies to control N pollution have been implemented on global, national and regional scales since the 1990s, with the nitrates directive being the main policy implemented in the European Union (EU; 91/676/EEC). At the same time an immense body of scientific work has been accumulated to provide N pollution mitigation strategies. Even though this work resulted in significant potential to reduce N deposition both in absolute values and per unit of product produced, the problem remains largely unsolved, especially in countries or regions with high N deposition (Erisman, 2021). When more extreme policies are implemented, a significant social opposition and conflict might rise. This became obvious in the Netherlands with the escalation of Dutch farmers' revolts in July 2022 (Volker, 2022). Even though the Netherlands is a particular case due to the high N use relative to the available area to be deposited, several countries are facing similar problems at least in areas overloaded with N. Therefore, N pollution mitigation strategies are still valid and needed. In this direction, nutritional strategies seem to be the most effective way to reduce N excretion at the animal level (Calsamiglia et al., 2010; Dijkstra et al., 2013). In the current communication, we will focus in describing a holistic nutritional management (HNM) scheme that leads to improved N use efficiency and reduced N excretion.

4.2. Contribution of dairy farming on nitrogen cascade

Nitrogen pollution of the environment has been described as an N cascade, which refers to the sequential transfer of reactive N (N_r) through

environmental systems and particularly to the transformations of N_r that enable it either to move from one system to another or to be stored within each system (Galloway et al., 2003). Non-reactive N (N_2) is converted to N_r naturally through microbial activity or by a lightning strike, and artificially by the Haber–Bosch process. Then it enters into the food chain where it is transformed, through biological processes, into inorganic forms of N such as ammonia (NH_3), ammonium (NH_4^+), nitrogen oxides (NO) and nitrous oxide (N_2O), and organic N such as urea and proteins (Galloway and Cowling, 2021). Even though the majority of anthropogenic N_r is introduced into the ecosystem in the form of fertilizers to support plant production, a considerable portion of crops and forages is consumed by animals. Of course, the role of ruminants is not negative since they have the ability to consume high fiber feeds and by-products of agricultural production, such as pasture, hay and straw, that are not digestible by humans and other non-herbivorous, and transform them to high-quality protein food for humans (Broderick, 2018). However, as in the case of non-ruminants, this biological process also results in forms of N_r that are excreted by the ruminant into the ecosystem. Therefore, dairy cattle farming is an indirect contributor of N cascade phenomena due to its role in the food chain.

To realize the impact of dairy farming in N cascade we need to consider that in the European Union (EU)-27 approximately 20.5 million dairy cows were farmed in 2020 (EUROSTAT, 2022). If we consider that each lactating cow consumes 480 g/day of N with a milk N use efficiency ($MNE = N \text{ in milk} / N \text{ intake; g/g}$) of 0.277 g/g (Huhtanen and Hristov, 2009), we may estimate that each cow excretes approximately 347 g/day of N in manure. Thus, assuming a 305 day/year lactating period it may be calculated that the EU-27 dairy sector,

without considering replacement heifers and the dry period of the dairy cows, consumes 3.00 and excretes 2.17 Tg of N per year. Leip et al. (2022) estimated a total of 20 Tg of virgin N_r entering the EU ecosystem every year, and 9.1 Tg of that plus 2.3 Tg of imported feed are being transferred into the livestock sector. Thus, it might be calculated that: (1) the overall involvement of lactating dairy cattle in N_r consumption is 26.3% of total N_r directed to animal feed, and (2) approximately 10% of the virgin N_r entering into the EU ecosystem is deposited as cattle manure. Certainly, the major part of this N_r in manure is used for further N_r transfer into plant protein but significant N_r transformations occur during manure storage and field application that move N_r to other systems contributing to the N cascade phenomenon. Several strategies have been developed to reduce these losses at the farm level (Soteriades et al., 2018). Moreover, Sutton et al. (2011) made it clear that a priority is to improve N use efficiency in crops and livestock. Considering that MNE of dairy cattle is relatively low but highly variable (Calsamiglia et al., 2010), and that a theoretical maximum is close to 0.43 g/g (Dijkstra et al., 2013), there is definitely much room for improvement and actual on-farm reduction of N_r moved to manure. Improved nutritional management may play an important role in this regard.

4.3. Holistic nutritional management

It is generally accepted that crude protein (CP) has been intentionally overfed to lactating dairy cows as a way to handle day-to-day variation in forages and farm practices (Firkins and Reynolds, 2005). Even though this practice has been criticized due to the detrimental effect on MNE, it was a suitable practice, especially for farms that couldn't control CP concentration of forages and concentrates. Feeding to requirement levels is not only a matter of

ration formulation, but it presupposes adequate farm feeding management since nutrition is the main factor defining cow's productivity. In this work, we are proposing an HNM scheme as the main strategy to feed animals to metabolizable protein (MP) requirements.

4.3.1. *Biological base*

In most dairy cattle feeding systems, protein requirements are estimated in the base of MP (Tedeschi et al., 2015), resulting in better accuracy compared with CP-based systems. Even though CP is still used as an indicator of N levels of a certain diet, it should not be used as the base for diet formulation. On the contrary, MP originating either from rumen microbes or from feed protein that escaped rumen degradation (Van Amburgh et al., 2015; NASEM, 2021) determines the actual amounts of amino acids (AA) that are available to the animal. As described by Russell et al. (1992), rumen bacteria may be categorized into those that ferment structural carbohydrates (Bact_{SC}) and those that ferment non-structural carbohydrates (Bact_{NSC}). These have different requirements in terms of N for their growth: Bact_{SC} require mainly NH₃ while Bact_{NSC} require less NH₃ and more free AA (Figure 4.1). Meeting their requirements will maximize microbial MP, while the excess of rumen NH₃ will be absorbed in the portal vein, converted to urea in the liver and then either recycled back to the rumen or, especially if protein is in excess, excreted in urine with a considerable cost in available energy (NASEM, 2021).

Van Amburgh et al. (2015) demonstrated that when metabolizable energy (ME) is the limited nutrient, the excess of N fed to a dairy cow is converted to urea by increasing urinary N (Figure 4.2). This is why several authors have suggested that strategies seeking to reduce N excretion should

target lowering urinary N and not necessarily faecal N (Dijkstra et al., 2011; Higgs et al., 2012). However, the inefficiency of rumen microbial protein synthesis depends mainly on the source of rumen available energy and protein. Therefore, from a nutritional perspective, dietary rations should be balanced primarily for maximizing rumen microbial protein synthesis by minimizing N losses in the rumen. Then, once rumen function is maximized, the cow should be supplied with feed protein digestible in the intestine to meet its protein requirements. The use of rumen-protected protein feed sources, such as soybean meal, canola meal, etc., and the provision of rumen protected AA might be beneficial for achieving this.

4.3.2. Feeding groups formation

It is well established that feeding one total mixed ration (TMR) to lactating cows in a herd will result in nutritional imbalances concerning cows producing lower or higher than the average of the herd. In contrast, several studies have established the economic benefits of group formation in a herd as a management practice that provides different diets to different groups, with the objective of feeding cows closer to their requirements (Kalantari et al., 2016; Wu et al., 2019). Moreover, St-Pierre and Thraen (1999) demonstrated, through Monte-Carlo simulation, that group formation may reduce N excretion per kg of milk produced by 8%, while Kalantari et al. (2016) estimated a 2.7% improvement of MNE when three feeding groups were formed instead of one. However, on a farm level it is not always easily acceptable to change nutrition practice from one feeding group to multiple ones, either due to requests from farm managers to 'keep it simple' (Contreras-Govea et al., 2015) or to actual

difficulties created by the design of farm facilities, available equipment, herd size and management ability (Dann, 2017).

Several criteria have been proposed to form feeding groups in dairy herds, ranging from simply milk yield or fat and protein milk yield, to cluster based on NEL and CP density requirements, with clustering being the most widely used. In these efforts, the environmental benefits became detectable; however, CP was used as the main indicator of protein requirements and, as mentioned already, this is not a suitable measure of N available to the cow. In a recent study, Barrientos-Blanco et al. (2020) used MP instead of CP, which resulted in 15.14 g/cow less N supply. Therefore, group formation may be the base on which diets maximizing MNE at different nutrient requirements are formulated.

4.3.3. Ration formulation

Formulation of the diet is the central element of HNM. It might refer to one feeding group or, preferably, to various feeding groups in a dairy herd. Before selecting a diet formulation system, it is necessary to have a scientific evaluation of the model, and taking into consideration the importance of improving MNE, that it is also evaluated for protein-limiting diets. However, it is common for only specific parts of the model to be evaluated, or for evaluation of overall performance without distinguishing for cases where ME or MP are the limiting factors. The evaluation of the latest version of the Cornell Net Carbohydrate and Protein System (CNCPS), however, with a dataset of 250 different TMR fed to lactating cattle was applied to the entire dataset (first limiting ME or MP allowable milk) and separately for diets where ME was the first limiting nutrient (first limiting ME allowable milk) and those where MP was

the first limiting nutrient (first limiting MP allowable milk). As demonstrated in Figure 4.3, the model performed best for MP limiting ($R^2_{\text{MDP}} = 0.82$, RMSPE = 1.1, CCC = 0.83), followed by the entire dataset ($R^2_{\text{MDP}} = 0.78$, RMSPE = 1.6, CCC = 0.83), and ME limiting diets ($R^2_{\text{MDP}} = 0.76$, RMSPE = 1.8, CCC = 0.84) (Van Amburgh et al., 2015). It became obvious that CNCPSv6.5 can be used to lower N intake levels of dairy cattle without impacting their production negatively. Thus, CNCPS v6.5 was selected as the main diet formulation model within the HMN approach. Therefore, the following guidelines on diet formulation are suggested:

Step 1. Define principal forages available to the farm (e.g. corn silage, alfalfa silage, alfalfa hay, wheat straw) and the desirable forage to concentrate ratio (F:C). This is calculated on a DM basis, and it might range from 40:60 to 60:40 for a high yielding group, and from 50:50 to 80:20 for a low yielding group.

Step 2. Select concentrate feeds that provide adequate levels of starch, sugars, NDF and N available both in the rumen and post-rumen. Nutrient recommendations for lactating Holstein cows by Cornell University are provided in Table 1. At this step ME and MP should not be balanced but to be close to targets.

Step 3. Rumen N balance. The CNCPSv6.5 provides a calculation of NH_3 requirements for microbial protein synthesis (peptide and NH_3 , % requirements). This calculation takes into consideration the degradation of protein sources in the rumen that provides peptides and NH_3 as well as the rumen degradation of energy sources (starch, sugars, NDF) to estimate MP derived from rumen bacteria. In an ideal situation, peptide and NH_3 (%)

requirements) should be 100%. However, depending on farm management and the detrimental effects that shortage of NH_3 has on microbial function in the rumen, it is recommended to be between 110 and 120% requirements. If peptide and NH_3 (% requirements) is higher than 120% it might be utilized by rumen microbes by providing additional energy. Otherwise, protein sources that provide soluble N in the rumen should be gradually reduced. The final goal of this step is to balance for rumen N and to maximize bacterial MP.

Step 4. ME and MP in balance. Once rumen function is maximized, to balance for MP requirements is necessary to add feeds that escape rumen degradation. Of course, the final inclusion of these feeds depends on the target of ME and MP (e.g. low or high group within the farm). It should be noted that several decisions in this step may provoke changes in the previous steps, especially in step 3. It is necessary to move back and forward several times in order to balance for both ME and MP within the nutritional limits described in Table 1.

Step 5. Amino acids, minerals and vitamins. Once MP and ME are in balance, attention should be given to AA balance, and especially to methionine and lysine. The CNCPSv6.5 provides calculations for both AA. In this direction, rumen protected methionine and lysine should be added to meet requirements. As reported by Van Amburgh et al. (2015) the optimal inclusion of Met and Lys is 2.6–2.8 and 6.8–7.0% MP, respectively. Last but certainly not least, attention should be given to vitamins and minerals.

4.3.4. Ration implementation

On a farm level we frequently observe the existence of three different TMR: (1) formulated TMR, (2) mixed TMR, and (3) consumed TMR. This

differentiation derives from several management factors at the farm level, and the principal objective of HNM is to unify these three TMR in one: the formulated diet.

Diet formulation with mechanistic models requires measured inputs to run the model. Among the most important are the body weight (BW) and body condition score (BCS) of the cows. The first influences a series of equations in the model, such as the prediction of dry matter intake (DMI), but the most important are related to the estimated ME and MP for maintenance. It is generally assumed that cow's BW is not significantly different among farms, and it may vary depending on the region and the genetic sources in each country. However, this is not true. Within the work of the Cowficiency project (Horizon2020- 777974; www.cowficiency.org) we reported an average BW of 730 ± 72 kg, with a range of average BW at the farm level from 662 to 783 kg. Furthermore, BCS is a good indicator of energy dynamics, and depending on group formation the BCS target will provide a measurable figure either of BCS loss that will support milk production or BCS gain that will increase ME and MP requirements. Therefore, both BW and BCS should be assessed frequently, and within the HNM the minimum length between two assessments was set at 3 months.

Another element that should be assessed frequently is the actual DMI. The CNCPSv6.5 provides both CNCPS and NRC equations to predict DMI, but both equations are empirical and contain significant error. Therefore, it is recommended to measure actual DMI frequently. Depending on group targets, cows may be fed either ad libitum or restricted. In the first case,orts should be 5% of the DM offer. Even though several farms weigh orts, they are not

assessing their DM. The correct measurement of DMI includes the assessment of both quantity (offered or refused) and DM content. Several studies have focused on the significant variation of feedstuff's chemical composition, and especially that of forages. St-Pierre and Weiss (2015) reported significant day-to-day variation in DM and starch of corn silage and in DM and NDF of alfalfa silage in dairy farms. Variation in DM of forages is crucial considering that diets are formulated on a DM basis, but mixed and offered on an as-fed basis. This is the main element of miscommunication between the nutritionist and the farmer. If actual DM of forages is not measured frequently, then the observed variation will create imbalances of ME and MP of the supplied TMR. Therefore, it is proposed that the DM of silages and other high moisture feedstuffs should be analyzed at least weekly, and the chemical composition of all ingredients fed to lactating dairy cattle once per month.

Another element of importance is the homogeneity of the TMR to reduce sorting by cattle. This is related to both mixing monitoring of the TMR and measures to increase its homogeneity. Oelber and Stone (2017) identified several factors in the TMR loading and mixing that may generate variation in the TMR. From an HNM perspective, the process needs to be monitored frequently by a specialist to detect errors in the mixing process, and samples of the TMR should be collected and analyzed with the Penn State Particle Separator and/or chemically to identify the extent of variation. Furthermore, measures to increase homogeneity of the TMR are needed during both diet formulation (e.g. inclusion of molasses, water, etc.) and mixing (particle size of forages, mixing order, time of mixing, etc.). To this end, there are available feed software programs that not only record daily mixing of the TMR but also have

the ability to adjust actual quantities based on the DM of forages. Even though feed software is used frequently to track the performance of a feeder, it may also be used as the basis to evaluate errors in weighing at each farm and to promote training of farm personnel responsible for feeding. Training of personnel is an essential element in any management scheme, and in this case the work of Oelber and Stone (2017) is an excellent guide for it.

Within the CowficieNcy project several dairy farms in Greece, Italy, Spain and the UK were evaluated for their N status, and a set of pilot farms applied the HNM scheme. Preliminary results suggest that MNE improvement is possible.

4.4. Conclusions

Anthropogenic N_r is an important element of modern food production systems defining the productivity of crops and livestock. However, it enters the ecosystem in such quantity that the ecosystems are overloaded with specific forms of N_r that cause pollution. Dairy cattle farming is a secondary contributor to the N cascade phenomenon as it transforms plant N_r to animal N_r . In this process, N_r transformation has a low efficiency due to necessary biological processes, such as maintenance protein turnover, and farm practices, such as feeding or manure management. In the current work, we describe an HNM scheme involving animal, feed and farm management to improve milk nitrogen use efficiency that consequently reduces N_r excretion in the environment.

4.5. References

- Barrientos-Blanco, J.A., White, H., Shaver, R.D., Cabrera, V.E., 2020. Improving nutritional accuracy and economics through a multiple ration-grouping strategy. *Journal of Dairy Science* 103, 3774-3785. doi: <https://doi.org/10.3168/jds.2019-17608>.
- Broderick, G.A., 2018. Review: Optimizing ruminant conversion of feed protein to human food protein. *Animal* 12, 1722-1734. doi: <https://doi.org/10.1017/S1751731117002592>.
- Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., Van Vuuren, A.M., 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184-1196. doi: <https://doi.org/10.1017/S1751731110000911>.
- Contreras-Govea, F.E., Cabrera, V.E., Armentano, L.E., Shaver, R.D., Crump, P.M., Beede, D.K., Vandehaar, M.J., 2015. Constraints for nutritional grouping in Wisconsin and Michigan dairy farms. *Journal of Dairy Science* 98, 1336-1344. doi: <https://doi.org/10.3168/jds.2014-8368>.
- Dan, H.M., 2017. Nutritional strategies for dry and fresh cows. In *Large dairy herd management* (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA, p. 669-712. doi: <https://doi.org/10.3168/ldhm.0632>.
- Dijkstra, J., Oenema, O., Bannink, A., 2011. Dietary strategies to reducing N excretion from cattle: implications for methane emissions. *Current Opinion in Environmental Sustainability* 3, 414-422. doi: <https://doi.org/10.1016/j.cosust.2011.07.008>.

- Dijkstra, J., Reynolds, C.K., Kebreab, E., Bannink, A., Ellis, J.L., France, J., Van Vuuren, A.M., 2013. Challenges in ruminant nutrition: towards minimal nitrogen losses in cattle. In Energy and protein metabolism and nutrition in sustainable animal production: 4th International Symposium on Energy and Protein Metabolism and Nutrition (ed. Oltjen, J.W., Kebreab, E., and Lapierre, H.), Wageningen: Wageningen Academic Publishers, pp. 47-58. doi: https://doi.org/10.3920/978-90-8686-781-3_3.
- Erisman, J.W., 2021. Setting ambitious goals for agriculture to meet environmental targets. *One Earth* 4, 15-18. doi: <https://doi.org/10.1016/j.oneear.2020.12.007>
- EUROSTAT, 2022. Bovine population - Dairy cows- annual data.
- Firkins, J.L., Reynolds, C.K., 2005. Whole-animal nitrogen balance in cattle. In Nitrogen and phosphorus nutrition of cattle: Reducing the environmental impact of cattle operations (ed. Pfeffer, E., Hristov, A.N.), CAB International, Wallingford, UK, pp. 167–186. <https://doi.org/10.1079/9780851990132.0167>.
- Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., Cosby, B.J., 2003. The nitrogen cascade. *BioScience* 53, 341-356. doi: [https://doi.org/10.1641/0006-3568\(2003\)053\[0341:Tnc\]2.0.Co;2](https://doi.org/10.1641/0006-3568(2003)053[0341:Tnc]2.0.Co;2).
- Galloway, J.N., Cowling, E.B., 2021. Reflections on 200 years of Nitrogen, 20 years later. *Ambio* 50, 745-749. doi: <https://doi.org/10.1007/s13280-020-01464-z>.

- Higgs, R.J., Chase, L.E., Van Amburgh, M.E., 2012. Case Study: Application and evaluation of the Cornell Net Carbohydrate and Protein System as a tool to improve nitrogen utilization in commercial dairy herds. *The Professional Animal Scientist* 28, 370-378. doi: [https://doi.org/10.15232/S1080-7446\(15\)30370-3](https://doi.org/10.15232/S1080-7446(15)30370-3).
- Huhtanen, P., Hristov, A.N., 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. *Journal of Dairy Science* 92, 3222-3232. doi: <https://doi.org/10.3168/jds.2008-1352>.
- Kalantari, A.S., Armentano, L.E., Shaver, R.D., Cabrera, V.E., 2016. Economic impact of nutritional grouping in dairy herds. *Journal of Dairy Science* 99, 1672-1692. doi: <https://doi.org/10.3168/jds.2015-9810>.
- Leip, A., Caldeira, C., Corrado, S., Hutchings, N.J., Lesschen, J.P., Schaap, M., De Vries, W., Westhoek, H., Van Grinsven, H.J.M., 2022. Halving nitrogen waste in the European Union food systems requires both dietary shifts and farm level actions. *Global Food Security* 35, 100648. doi: <https://doi.org/10.1016/j.gfs.2022.100648>.
- NASEM, 2021. Nutrient requirements of dairy cattle, 8th revised edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/25806>.
- Oelber, T.J., Stone, W.C., 2017. Total mixed rations and feed delivery system. In *Large dairy herd management* (ed. Beede, D.K.), American Dairy Science Association, Champaign, Illinois, USA, p. 751-769. doi: <https://doi.org/10.3168/ldhm.0632>.

- Russell, J.B., O'Connor, J.D., Fox, D.G., Van Soest, P.J., Sniffen, C.J., 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *Journal of Animal Science* 70, 3551-3561. doi: <https://doi.org/10.2527/1992.70113551x>.
- Soteriades, A.D., Gonzalez-Mejia, A.M., Styles, D., Foskolos, A., Moorby, J.M., Gibbons, J.M., 2018. Effects of high-sugar grasses and improved manure management on the environmental footprint of milk production at the farm level. *Journal of Cleaner Production* 202, 1241-1252. doi: <https://doi.org/10.1016/j.jclepro.2018.08.206>.
- St-Pierre, N.R., Thraen, C.S., 1999. Animal grouping strategies, sources of variation, and economic factors affecting nutrient balance on dairy farms. *Journal of Animal Science* 77(suppl_2), 72-83. doi: https://doi.org/10.2527/1999.77suppl_272x.
- St-Pierre, N.R., Weiss, W.P., 2015. Partitioning variation in nutrient composition data of common feeds and mixed diets on commercial dairy farms. *Journal of Dairy Science* 98, 5004-5015. doi: <https://doi.org/10.3168/jds.2015-9431>.
- Sutton, M.A., van Grinsven, H., Billen, G., Bleeker, A., Bouwman, A.F., Bull, K., Erisman, J.W., Grennfelt, P., Grizzetti, B., Howard, C.M., Oenema, O., Spranger, T., Winiwarter, W., 2011. Summary for policy makers. In *The European Nitrogen Assessment: Sources, effects and policy perspectives* (ed. Bleeker, A., Grizzetti, B., Howard, C.M., Billen, G., van Grinsven, H., Erisman, J.W., Sutton, M.A. and Grennfelt, P.), Cambridge

University Press, Cambridge, p. xxiv-xxxiv. doi:
<https://doi.org/10.1017/CBO9780511976988.002>.

Tedeschi, L.O., Fox, D.G., Fonseca, M.A., Cavalcanti, L.F.L., 2015. Models of protein and amino acid requirements for cattle. *Revista Brasileira de Zootecnia* 44, 109-132. doi: <https://doi.org/10.1590/S1806-92902015000300005>.

Van Amburgh, M.E., Collao-Saenz, E.A., Higgs, R.J., Ross, D.A., Recktenwald, E.B., Raffrenato, E., Chase, L.E., Overton, T.R., Mills, J.K., Foskolos, A., 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. *Journal of Dairy Science* 98, 6361-6380. doi: <https://doi.org/10.3168/jds.2015-9378>.

Volker, R., 2022. Dutch farmers revolt. *Guardian* (Sydney) 2026, 10-10.

Wu, Y., Liang, D., Shaver, R.D., Cabrera, V.E., 2019. An income over feed cost nutritional grouping strategy. *Journal of Dairy Science* 102(5), 4682-4693. doi: <https://doi.org/10.3168/jds.2018-15302>.

4.6. Tables and Figures

Table 4.1. Basic nutrient recommendations for feed formulation with the Cornell Net Carbohydrate and Protein System (Van Amburgh, M.E., Overton, T.R., and Chase, L.E., Cornell University).

Item ¹	Units	Fresh	High producing
		cow	cow
DM intake	kg/day	> 19	> 23
ME allowable milk	% requirements	80-90	100-110
MP allowable milk	% requirements	90-105	100-110
MP from bacteria	% MP	> 40	> 40
Rumen NH ₃ balance	% requirements	110-120	110-120
Urea cost	Mcal/day	< 0.5	< 0.5
Forage NDF	% Body Weight	0.8 – 0.9	0.9-1.0
Total NDF	% DM	28-35	28-35
Starch	% DM	22-28	24-28
Sugars	% DM	4-7	4-7
Fat	% DM	4.0-4.5	4.5-5.0
Methionine	% MP	2.6-2.8	2.6-2.8
Lysine	% MP	6.8-7.0	6.8-7.0

¹ DM: dry matter, ME: Metabolizable Energy, MP: Metabolizable Protein, NDF: Neutral detergent fibre

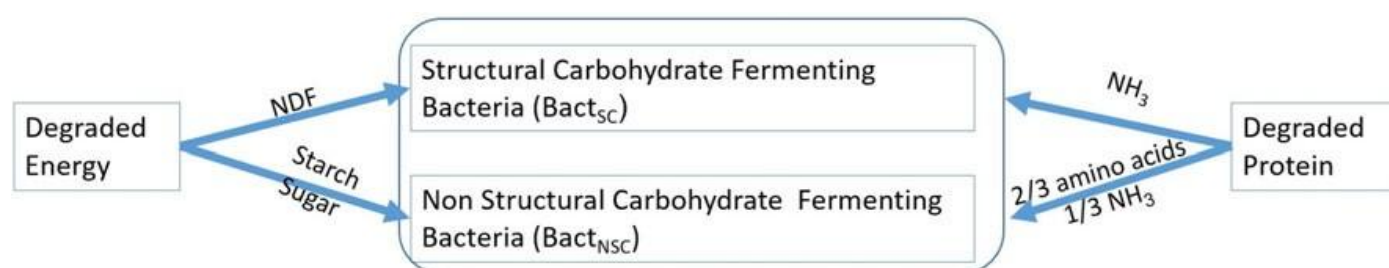


Figure 4.1. Nutrient requirements of rumen bacteria that ferment structural carbohydrates (Bact_{SC}) and those that ferment non-structural carbohydrates (Bact_{NSC}).

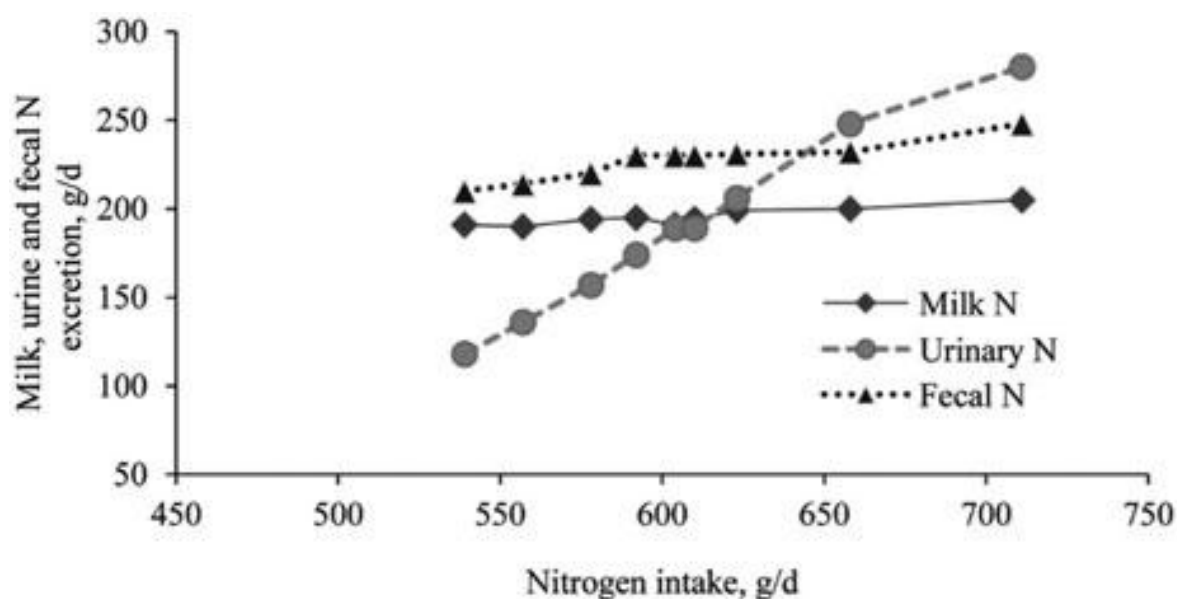


Figure 4.2. Nitrogen excretion in milk, faeces, and urine based on nitrogen intake in lactating dairy cattle under controlled conditions of energy as first limiting (Van Amburgh et al., 2015).

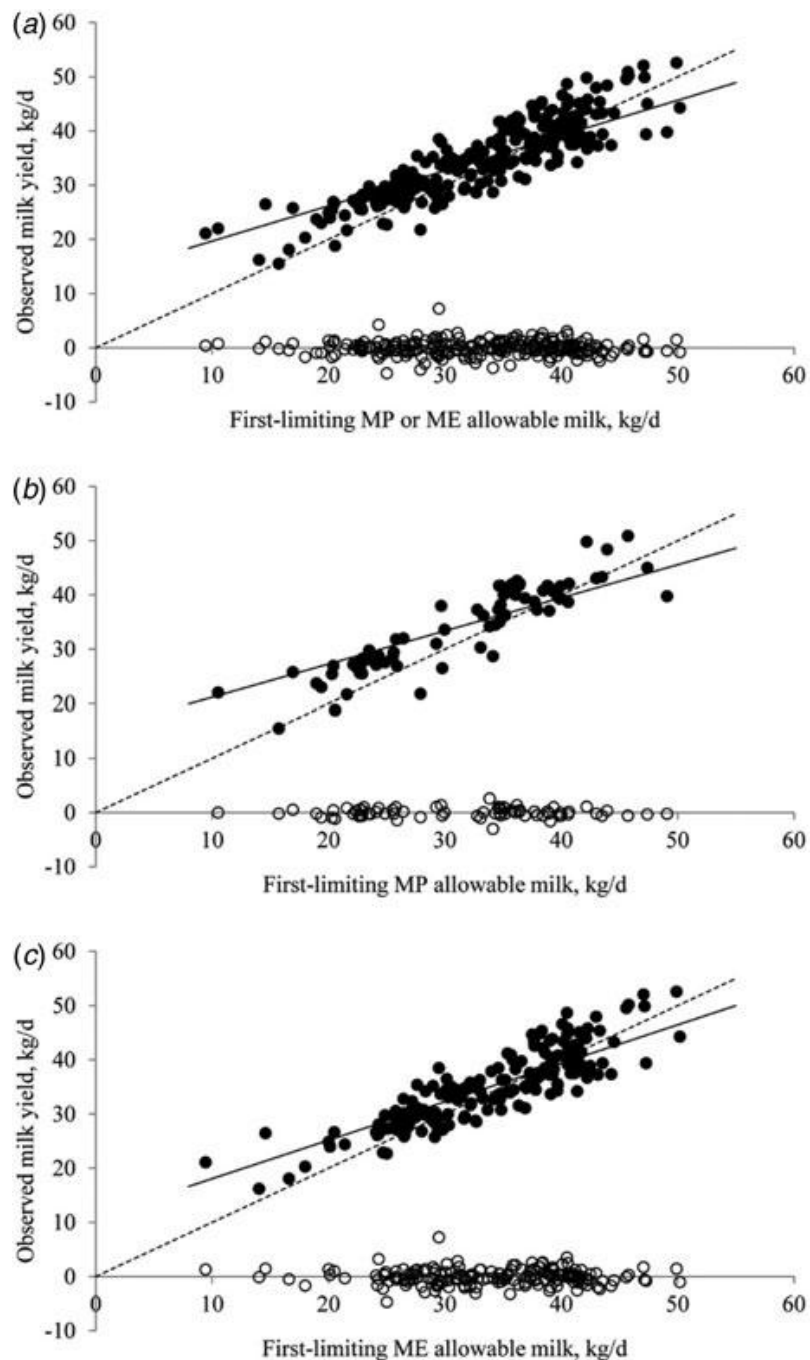


Figure 4.3. Observed versus Cornell Net Carbohydrate and Protein System predictions (●) for (A) first-limiting MP- or ME allowable milk ($y = 0.65x + 13.17$), (B) MP first-limiting milk ($y = 0.61x + 15.06$), and (C) ME first-limiting milk ($y = 0.71x + 10.92$). Mixed model residuals are also shown on the graph (○). (Van Amburgh et al., (2015)).

Chapter 5

General Discussion

5.1. Introduction

The public concern about environmental issues has been increased over the last decades (Xu et al., 2022). Nitrogen (N) pollution is responsible for multiple polluting phenomena, such as terrestrial and aquatic eutrophication and soil acidification (Sutton et al., 2011). Human activities, related to agricultural practices have disrupted the N cycle, creating the so-called N cascade phenomenon (Galloway et al., 2003; Galloway et al., 2008). As a consequence, inorganic forms of N (ammonia, NH_3 ; ammonium, NH_4^+ ; nitrogen oxides, NO_x ; nitrous oxide, N_2O etc.) are formed and contribute not only to N pollution, but also to air quality degradation, formation of particulate matter (PM) and greenhouse gas (GHG) emissions (Place and Mitloehner, 2010; Sutton et al., 2011). Dairy cattle farming is considered one of the main contributors regarding NH_3 emissions (Malherbe et al., 2022). Manure applied on fields, on farm manure management and surplus N supplied to animals contributes to NH_3 deposition into the ecosystem (Bougouin et al., 2016). Within European Union (EU) agriculture is responsible for 94 % of total NH_3 emissions, with cattle accounting for 51.3 % of total NH_3 emissions (Malherbe et al., 2022). As the contribution of cattle to N pollution is not negligible, dairy sector needs to develop strategies for the increase of nitrogen use efficiency (NUE) at a farm and animal level in order to minimize N losses (Calsamiglia et al., 2010; Sutton et al., 2011).

Within dairy systems, dairy replacement heifers are usually overlooked when it comes to N pollution deriving from the dairy sector. However, heifers are an import part of dairy herds, replacing culled cows, offering the opportunity to increase herd size and improving herd's genetic potential. In Table 1.1,

replacement rates from different countries and rearing systems were presented, leading to an average replacement rate of 30.8 %, ranging from 21.3 to 40.0% (Maher et al., 2008; Alvåsen et al., 2018). This, practically, means that 30.8 cows need to be replaced in the milking herd, annually. However, in reality, the annual heifer number that a dairy farm needs to maintain herd size is greater, as other parameters should be taken into consideration, such as the age at first calving (AFC) and heifer mortality rate. For example, a 100-cow dairy farm with AFC at 24 months, 30.8 % replacement rate and 6.4 % mortality rate, requires annually 32.8 heifers without, although, considering a safety cushion. Moreover, considering a 2-year rearing cycle, 65.6 heifers are required biannually. Therefore, it is evident that heifer herd size of a farm is not negligible, with the associated rearing costs and potential N losses, impacting economic and environmental sustainability of the farm.

Rearing management practices of replacement heifers appear to impact overall farm N losses (Foskolos and Moorby, 2018). More specifically, it has been found that there is a negative correlation between AFC and lifetime N use efficiency (LNE; $r = -0.15$), while AFC was the factor that negatively impacted the most the NUE of replacement heifers ($r = -0.86$). The negative impact of AFC on NUE efficiency can be portrayed by the following example. Based on available published data, it was calculated that heifers of 9 to 17 months old contribute to NH₃ emissions on average 17.3 g/d (Lascano et al., 2008; Powell et al., 2008). Taking into consideration that a 100-cow dairy farm with average AFC at 22, 24 or 26 months requires 33.2, 36.2 and 39.2 heifers, respectively, this results in NH₃ emissions of 209.6, 228.6 and 247.5 kg/year at a herd level (Plomaritou et al., 2025). Based on a metanalysis conducted by Bougouin et al.

(2016), a cow on average emits 60.1 g/d of NH_3 (ranging from 7.8 to 213 g/d). Thus, NH_3 emissions of the 100-cow herd can be estimated at 2193.7 kg/year, resulting in 2403.0, 2422.1 and 2441.1 kg/year of NH_3 in total, when AFC is at 22, 24 and 26 months, respectively. As a consequence, heifers account for the 8.6 %, 9.4 % and 10.2 % of total farm NH_3 emissions, when AFC is at 22, 24 and 26 months, respectively.

Therefore, improving N utilization of dairy replacement heifers might increase overall farm NUE. Our aim was to identify factors that impact and improve NUE of replacement heifers and towards this direction 3 studies were conducted with specific objectives.

5.2. Theoretical Approach of the Thesis

As a first step, the review of the literature (Chapter 1) aimed to provide all information related to heifer rearing management that possibly could affect NUE. A main goal of all rearing programs is to produce good quality heifers with a relatively low AFC. Across all rearing systems AFC at 24 months has been established, as an age that does not compromise growth and improves first and lifetime milk production (Wathes et al., 2014; Eastham et al., 2018). The AFC does not only affect productivity, but also has been found to be negatively correlated with farm NUE (Foskolos and Moorby, 2018). It has been reported that higher N losses are occurring with extended rearing periods, resulting in a decrease of overall farm NUE (Foskolos and Moorby, 2018). Therefore, AFC has been identified as one of the most important parameters that affect both farm NUE and dairy replacement NUE.

For the achievement of desired AFC, growth management plays a crucial role. During this approximately 2-year rearing process, heifers are reaching maturity and this is inevitable. However, at specific growth benchmarks, it is likely that heifers have not attained the proper body size that will enable them to achieve an AFC at 24 months. In other words, the resulting AFC is a combination of age, height and body weight (BW) at the age of breeding (Wathes et al., 2014). As a consequence, growth management should be focused on the attainment of proper body size at growth benchmarks, such as the age of breeding, that is considered to be at 14 months. In order to do so, herd's mature body weight (MBW) is a prerequisite that needs to be determined (Van Amburgh et al., 2019). Growth targets of 55 % MBW at age of first breeding and 85 % MBW at AFC are used as a tool to project heifer growth and adjust rearing program (NASEM, 2021). In addition to projecting growth and setting growth targets, frequent BW monitoring and adjustment of ADG when required are important to achieve growth targets and ultimately desired AFC. Hence, growth was an additional factor that has been identified to indirectly impact NUE.

It should be kept in mind, that heifer rearing is a dynamic process and is directly affected by growth. During the rearing period, body composition, and as a consequence energy and protein requirements, are changing depending on growth stages (Van Amburgh et al., 2019). Heifers in early life stage until puberty have increased protein requirements compared to older heifers (NASEM, 2021). Hence, N utilization is expected to be higher for younger heifers. From a physiological point of view there is a threshold of N utilization response (Van Amburgh et al., 2019). Therefore, based on animal's

physiological and metabolism mechanisms, as well as diet adjustments according to each growth stage's requirements, NUE can be maximized, while N losses can be minimized. Towards this direction, there is evidence that the effects of diet and especially the protein level supplied to animals affects N excreted in the environment (Dijkstra et al., 2013; Bougouin et al., 2016). Therefore, CP level was identified as a possible factor, whose adjustment might reduce N losses.

Moreover, ruminants have the intrinsic ability to incorporate N available in the rumen into microbial protein (Schwab et al., 2005). Microbial protein and feed protein that escapes rumen degradation consist the main metabolizable protein (MP) sources for the animal (Russell et al., 1992). The rumen degradable protein (RDP) is the main N supplier to microbial protein synthesis. Once, microbial N demands are met, RUP may further improve N utilization (Tomlinson et al., 1997). Consequently, the RDP:RUP ratio was considered another factor, whose alteration may further improve NUE. Thus, rumen undegradable protein (RUP) sources are also required to meet AA requirements of growing heifers (Storm and Orskov, 1983; Lascano et al., 2016).

As a conclusion, among all the factors that were identified to impact NUE there is distinguished an interdependency and interaction. The efficiency of reproduction (AFC), growth (BW monitoring, ADG) and nutritional management (CP, RDP and MP level, energy supply) determine one another. Management practices impact this continuous flow of events and therefore affect NUE of replacement heifers.

Therefore, as a second step it was interesting to look into conditions of heifer rearing and identify factors that affect NUE at a farm level (Chapter 2). Thus, we investigated the current status of NUE of dairy replacement heifers reared under Mediterranean conditions, we evaluated nutritional management taking into consideration reproduction parameters and defined possible areas for improvement.

The third step was to examine the effects of protein supply to NUE of replacement heifers (Chapter 3) and we used two approaches. We conducted two digestibility trials: in Trial 1, we focused on the effects of different levels of CP on NUE. In Trial 2, we maintained the same low CP level and altered the RDP level supplied to heifers. Therefore, we aimed to have a comprehensive overview about N digestibility and partitioning based solely on protein availability and degradability.

Lastly, within the framework of Cowficiency project (Horizon2020 - 777974; <http://www.cowficiency.org>), visits at dairy farms took place with the main aim to describe nutritional management practices regarding both dairy cows (Mikenzie's Hanlon PhD thesis) and dairy replacement heifers (current PhD thesis) and define possible areas for NUE improvement. Therefore, the final step was to gather all information obtained and transmute them into a guide (Chapter 4).

5.3. A multi-gear nutritional plan for heifers based on metabolizable protein requirements should be implemented to reduce N losses

The field study was an effort to describe the reality and management practices implemented in modern heifer rearing in Greece. In the current study,

we approached 25 farms and based on their acceptability we evaluated 14 farms. Data collection was accomplished through a 2-day visit on each farm. We built three datasets: reproduction, growth and nutritional management dataset, where 2453 cows from 12 herds, 643 heifers from 13 herds, and rations from 10 herds were included, respectively.

We found that heifers calved for first time in greater age than 24 months. However, this delay was not related to nutritional limitations, rather to growth management practices, mainly due to the lack of BW monitoring. In particular, we observed that heifers at 14.3 months had attained the proper BW (407.9 kg; Table 2.3) and had achieved the set BW target of 55 % MBW estimated 14.3 months (403.4 kg; Table 2.3). Heifers, though, were later introduced into reproduction process at 15.4 months weighing 437.1 kg. Regarding nutritional management, when CP levels were examined at a farm level, there were cases where CP level was rather low or considerably high (8.4-16.7 % DM; Table 2.4). When CP levels were examined per age group, it appeared that CP levels were moderate (12.2-14.6 % DM; Table 2.5). Nevertheless, we observed that even though CP levels were within recommendations per age group, MP was supplied above requirements in all age groups of heifers, ranging from 103 to 133 % of MP requirements for 6 and 24 months heifers, respectively (Table 2.6), with surplus MP being excreted into the environment. As a conclusion, nutritional plan should be implemented based on each stage's requirements and attention should be given at the protein sources. Moreover, the use of mechanistic nutritional models (e.g. CNCPS) is a useful tool to prevent any nutrient loss.

However, some limitations of the study should be discussed. Firstly, a measuring tape instead of a scale was used. However, the lack of weight scale was a common phenomenon in all dairy farms visited. One of our main conclusions was that the lack of BW monitoring led to poor growth and reproduction management, and finally to a higher AFC. Therefore, under no ideal conditions, measuring tape could be proven a useful tool in overall heifer management. The high correlation that has been detected between heart girth circumference (HGC) measurements and heifer BW (Heinrichs et al., 1992; Heinrichs et al., 2007) allows the use of a measuring tape when a scale does not exist and enables decision making regarding growth management. Secondly, forage variation was not included in the current field study and it was considered that diets remained the same during this 2-year rearing process. Following 14 farms for 2 years is unrealistic and sources (humane and financially) consuming. Most studies in the literature were based on large national databases without farm visits to evaluate growth in relation to reproduction parameters (e.g. Cue et al., 2012), without taking into consideration nutritional factors. To our knowledge there is no other field study that evaluated both heifer growth and NUE at a farm level. Thus, our strategy was to take a “screenshot” of management practices and NUE by the time of visiting and to define possible areas for improvement. After each visit, a report with suggestions and improvements was made for each individual farm. Therefore, whether farms implemented or not the improvements regarding management was beyond the scope of this study and to address this issue further research is needed. However, we strongly believe that even considering these limitations, the current assessment study is useful for all stakeholders.

5.4. A lower crude protein and rumen degradable protein level may be beneficial on NUE of replacement heifers

Even though, there is plenty of information and data regarding N utilization of dairy cows (Batista et al., 2017; Bougouin et al., 2022), this is not the case in dairy heifers. Regarding N partitioning in replacement heifers a reasonable merit of available research had been focused on limit feeding and forage to concentrate (F:C) ratio (Hoffman et al., 2007; Zanton and Heinrichs, 2009; Kruse et al., 2010; Lascano et al., 2016; Zanton and Heinrichs, 2016), or testing the effect of different types of forages on N excretion (Su et al., 2017; Corea et al., 2020). Therefore, we aimed to investigate N allocation in dairy heifers without constraining DMI (limit feeding) or testing different F:C ratios or forage types and simulate real farm practices. Additionally, from the first study it was evident that in all heifer groups MP was oversupplied. Thus, as a next step, we conducted two N digestibility trials to examine the effects of protein source, testing two different CP and RDP levels (Trial 1 and Trial 2, respectively), on N balance and excretion.

In Trial 1, we observed that N balance for heifers fed the higher CP level (HCP) was greater than heifers fed the lower CP level (LCP; 48.3 and 34.8 g/d for HCP and LCP, respectively; $P = 0.03$; Table 3.6), whereas NUE was not affected by the treatments. Even though the higher CP level benefited N balance, it led to increased ruminal N (12.6 and 8.1 mg/dL for HCP and LCP, respectively; $P = 0.03$; Table 3.8) and urinary N concentration (60.8 and 43.1 g/d for HCP and LCP, respectively; $P < 0.01$; Table 3.8), meaning a higher N loss in the environment. In Trial 2, under the same low CP level, heifers fed a lower RDP level (LRDP) compared with a higher RDP level (HRDP) exhibited

a tendency for increased NUE (23.0 and 17.4 % for LRDP and HRDP, respectively; $P = 0.10$; Table 3.7) and reduced N excretion via urine (75.1 and 85.8 g/d for LRDP and HRDP, respectively; $P = 0.10$; Table 3.7).

Our results indicated that a higher CP level, although benefited N balance, did not affected NUE and increased urinary N excretion. Thus, a lower CP level fits well in a nutritional plan focused to reduce N excretion and minimize environmental impact of dairy heifers. Moreover, we detected a tendency for increased NUE and lower N concentration in the urine with the lower RDP level. Diets of replacement heifers usually contain lower quality feeds, yet, our results highlight the importance of using products that escape ruminal degradation when efforts are made to improve N utilization and reduce N excretion of replacement heifers.

A limitation of the study could possibly be the low number of animals. Maybe this is the reason why in Trial 2, we did not report significant different results, but tendencies. Recent studies have utilized a large number of animals in order to examine the effects of protein supply on N excretion. For instance, Corea et al. (2020) and Johansen et al. (2022) utilized 32 and 36 heifers, respectively. In our opinion, this is in contrast with the golden rule of research summarized by the 3 R's principle; replacement, reduction and refinement (Russell et al., 1959; NRC, 2011). Thus, as a first step, we conducted a power analysis with the objective to use the lowest number of animals possible and still detect significant differences on N utilization.

Moreover, both studies used spot sampling technique for feces and urine collection. Johansen et al., used 2 different sampling points for 2 days (1 for

each day) for urine sampling and 2 time points for 4 days (1 for every 2 days) for feces sampling, while total duration of experiment was 42 days, in 3 experimental periods. In contrast, the experimental study of Corea et al, progressed in 10 weeks (3 experimental periods) and used more sampling points, yet sampling was limited in a 10-hour window, from 0700 to 1700h. Both studies failed to follow a sampling scheme that represents the 24-hour digestion cycle, rendering the quality of published results questionable. Thus, in our opinion the level of detail and intervention of current study, even though tendencies were detected, provides data with less level of uncertainty.

Therefore, considering our results these digestibility trials can be used as preliminary research that identifies parameters for further research. As a next step, a greater number of animals can be used for a N balance study, examining N supply and its impact on growth.

5.5. Holistic nutritional management, a main strategy to improve farm NUE

The final study of this PhD thesis was focused on holistic nutritional management (HNM). Traditionally, overfeeding of CP was a strategy to address the variation in forages (Firkins and Reynolds, 2005). However, within the work of the CowficieNcy project, during farm visits regarding Mikenzie's Hanlon PhD thesis focused on dairy cows and current thesis focused on dairy replacement heifers, we observed that feeding protein above requirements was a common problem in many cases, deriving mainly from general nutritional management of the farms.

Therefore, our main goal was to create a step by step nutritional guide. The purpose of this guide was dual. Firstly, to depict all possible factors interacting and impacting NUE. A second goal, but equally important was to enable all stakeholders (farmers, nutritionists, farm managers) to improve NUE at a farm and animal level through changes in nutritional and management practices.

Even though the (HNM) scheme has been focused mainly on dairy cows, a holistic plan includes also replacement heifers. As it has been shown from the current work, improving overall farm NUE is not feasible without proper heifer management. Therefore, the management practices described in Chapter 4 are equally addressed to dairy cows and heifers.

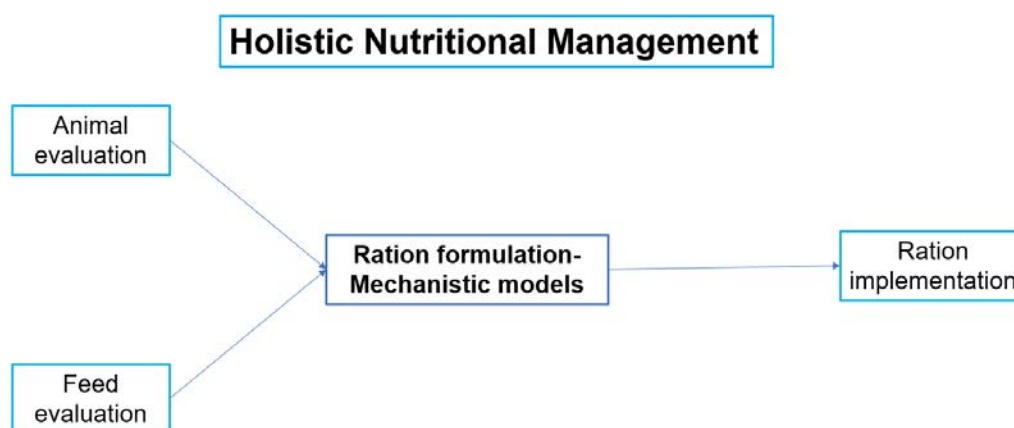


Figure 5.1. Important inputs and course of action under the holistic nutritional management approach.

As shown in Figure 5.1, the HNM approach includes farm specific variables. The assessment of animal (BW, body condition score, dry matter intake) and feed (chemical analysis) related factors are important inputs into the mechanistic nutritional model, with the objective to characterize the herd and

ingredients available for a proper ration formulation. Moreover, the selection of the right mechanistic model itself, is of extremely high importance. When it comes to minimize N losses, the the Cornell Net Protein and Carbohydrate System (CNCPS) has been proven a useful tool, due to its sensitivity in low protein diets, especially when MP is the first limiting factor (Van Amburgh et al., 2015). Lastly, frequent monitoring of mixing and loading processes, ensuring diet homogeneity and personnel training are key actions towards a proper diet implementation.

5.6. Conclusions

This PhD dissertation was focused on identifying factors affecting NUE of dairy replacement heifers and define possible areas for improvement. The main conclusions are:

- i) Considering a balanced diet as a prerequisite, the frequent monitoring of heifers' BW is an essential management tool to ensure the attainment of proper body size at first calving.
- ii) The trend that NUE followed suggested that lower CP level of older heifer diets, may reduce N excretion and increase NUE.
- iii) Nutritional mechanistic models that balance rations on MP basis allow precision feeding and prevent potential nutrient losses.
- iv) The higher CP level affected positively N balance, however in the expense of higher N concentration in ruminal environment and urine. As a consequence, higher N losses in the environment were observed.

v) A lower CP level had the same effects on NUE and lower N excreted in the environment than the higher CP level. Thus, a lower CP level is beneficial to N losses reduction.

vi) When feeding a low CP diet, a lower RDP protein level improves NUE and reduces urinary N excretion.

vii) The HNM scheme is a tool to reduce N excretion in the environment, taking into consideration animal, feed and farm management and improving milk nitrogen use efficiency.

5.7. References

- Alvåsen, K., Dohoo, I., Roth, A., Emanuelson, U., 2018. Farm characteristics and management routines related to cow longevity: A survey among swedish dairy farmers. *Acta Veterinaria Scandinavica* 60, 38. doi: <https://doi.org/10.1186/s13028-018-0390-8>.
- Batista, E.D., Detmann, E., Valadares Filho, S.C., Titgemeyer, E.C., Valadares, R.F.D., 2017. The effect of CP concentration in the diet on urea kinetics and microbial usage of recycled urea in cattle: A meta-analysis. *Animal* 11, 1303-1311. doi: <https://doi.org/10.1017/S1751731116002822>.
- Bougouin, A., Hristov, A., Dijkstra, J., Aguerre, M.J., Ahvenjärvi, S., Arndt, C., Bannink, A., Bayat, A.R., Benchaar, C., Boland, T., Brown, W.E., Crompton, L.A., Dehareng, F., Dufrasne, I., Eugène, M., Froidmont, E., van Gastelen, S., Garnsworthy, P.C., Halmemies-Beauchet-Filleau, A., Herremans, S., Huhtanen, P., Johansen, M., Kidane, A., Kreuzer, M., Kuhla, B., Lessire, F., Lund, P., Minnée, E.M.K., Muñoz, C., Niu, M., Nozière, P., Pacheco, D., Prestløkken, E., Reynolds, C.K., Schwarm, A., Spek, J.W., Terranova, M., Vanhatalo, A., Wattiaux, M.A., Weisbjerg, M.R., Yáñez-Ruiz, D.R., Yu, Z., Kebreab, E., 2022. Prediction of nitrogen excretion from data on dairy cows fed a wide range of diets compiled in an intercontinental database: A meta-analysis. *Journal of Dairy Science* 105, 7462-7481. doi: <https://doi.org/10.3168/jds.2021-20885>.
- Bougouin, A., Leytem, A., Dijkstra, J., Dungan, R.S., Kebreab, E., 2016. Nutritional and environmental effects on ammonia emissions from dairy cattle housing: A meta-analysis. *Journal of Environmental Quality* 45, 1123-1132. doi: <https://doi.org/10.2134/jeq2015.07.0389>.

- Calsamiglia, S., Ferret, A., Reynolds, C.K., Kristensen, N.B., van Vuuren, A.M., 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4, 1184-1196. doi: <https://doi.org/10.1017/S1751731110000911>.
- Corea, E.E., Castro-Montoya, J., Mendoza, M.V., López, F.M., Martinez, A., Alvarado, M.E., Moreno, C., Broderick, G.A., Dickhoefer, U., 2020. Effect of forage source and dietary rumen-undegradable protein on nutrient use and growth in dairy heifers. *Animal Feed Science and Technology* 269, 114658. doi: <https://doi.org/10.1016/j.anifeedsci.2020.114658>.
- Cue, R.I., Pietersma, D., Lefebvre, D., Lacroix, R., Wade, K., Pellerin, D., Passillé, A.-M.d., Rushen, J., 2012. Growth modeling of dairy heifers in québec based on random regression. *Canadian Journal of Animal Science* 92, 33-47. doi: <https://doi.org/10.4141/cjas2011-083>.
- Dijkstra, J., Oenema, O., van Groenigen, J.W., Spek, J.W., van Vuuren, A.M., Bannink, A., 2013. Diet effects on urine composition of cattle and n₂o emissions. *Animal* 7, 292-302. doi: <https://doi.org/10.1017/S1751731113000578>.
- Eastham, N.T., Coates, A., Cripps, P., Richardson, H., Smith, R., Oikonomou, G., 2018. Associations between age at first calving and subsequent lactation performance in uk holstein and holstein-friesian dairy cows. *Plos One* 13, e0197764. doi: <https://doi.org/10.1371/journal.pone.0197764>.
- Firkins, J.L., Reynolds, C.K., 2005. Whole-animal nitrogen balance in cattle. In *Nitrogen and phosphorus nutrition of cattle: Reducing the environmental impact of cattle operations* (ed. Pfeffer, E., Hristov, A.N.), CAB

- International, Wallingford, UK, pp. 167–186.
<https://doi.org/10.1079/9780851990132.0167>.
- Foskolos, A., Moorby, J.M., 2018. Evaluating lifetime nitrogen use efficiency of dairy cattle: A modelling approach. *Plos One* 13, e0201638. doi: <https://doi.org/10.1371/journal.pone.0201638>.
- Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., Cosby, B.J., 2003. The nitrogen cascade. *BioScience* 53, 341-356. doi: [https://doi.org/10.1641/0006-3568\(2003\)053\[0341:Tnc\]2.0.Co;2](https://doi.org/10.1641/0006-3568(2003)053[0341:Tnc]2.0.Co;2).
- Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* 320, 889. doi: <https://doi.org/10.1126/science.1136674>.
- Heinrichs, A.J., Erb, H.N., Rogers, G.W., Cooper, J.B., Jones, C.M., 2007. Variability in holstein heifer heart-girth measurements and comparison of prediction equations for live weight. *Preventive Veterinary Medicine* 78, 333-338. doi: <https://doi.org/10.1016/j.prevetmed.2006.11.002>.
- Heinrichs, A.J., Rogers, G.W., Cooper, J.B., 1992. Predicting body weight and wither height in holstein heifers using body measurements. *Journal of Dairy Science* 75, 3576-3581. doi: [https://doi.org/10.3168/jds.S0022-0302\(92\)78134-X](https://doi.org/10.3168/jds.S0022-0302(92)78134-X).
- Hoffman, P.C., Simson, C.R., Wattiaux, M., 2007. Limit feeding of gravid holstein heifers: Effect on growth, manure nutrient excretion, and

- subsequent early lactation performance. *Journal of Dairy Science* 90, 946-954. doi: [https://doi.org/10.3168/jds.S0022-0302\(07\)71578-3](https://doi.org/10.3168/jds.S0022-0302(07)71578-3).
- Johansen, M., Madsen, P.A., Brask-Pedersen, D.N., Lund, P., 2022. Effect of age and dietary crude protein level on nitrogen excretion in dairy heifers. *Livestock Science* 264, 105058. doi: <https://doi.org/10.1016/j.livsci.2022.105058>.
- Kruse, K.A., Combs, D.K., Esser, N.M., Coblenz, W.K., Hoffman, P.C., 2010. Evaluation of potential carryover effects associated with limit feeding of gravid holstein heifers¹. *Journal of Dairy Science* 93, 5374-5384. doi: <https://doi.org/10.3168/jds.2010-3401>.
- Lascano, G.J., Koch, L.E., Heinrichs, A.J., 2016. Precision-feeding dairy heifers a high rumen-degradable protein diet with different proportions of dietary fiber and forage-to-concentrate ratios. *Journal of Dairy Science* 99, 7175-7190. doi: <https://doi.org/10.3168/jds.2016-11190>.
- Lascano, G.J., Zanton, G.I., Moody, M.L., Topper, P.A., Wheeler, E.F., Heinrichs, A.J., 2008. Short communication: Effect of changing the ratio of forage to concentrate on ammonia emissions by dairy heifers¹. *Journal of Dairy Science* 91, 4301-4306. doi: <https://doi.org/10.3168/jds.2008-1176>.
- Maher, P., Good, M., More, S.J., 2008. Trends in cow numbers and culling rate in the irish cattle population, 2003 to 2006. *Irish Veterinary Journal* 61, 455. doi: <https://doi.org/10.1186/2046-0481-61-7-455>.
- Malherbe, L., German, R., Couvidat, F., , Zanatta, L., Blannin, L., James, A., Létinois, L., Schucht, S., Berthelot, B. and Raoult, J., 2022. Emissions of ammonia and methane from the agricultural sector. Emissions from

livestock farming (ETC Report 2022/21). European Topic Centre on Human Health and the Environment.

NASEM, 2021. Nutrient requirements of dairy cattle, 8th revised edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/25806>.

NRC, 2011. Guide for the care and use of laboratory animals: Eighth edition. The National Academies Press, Washington, DC. doi: <https://doi.org/10.17226/12910>.

Place, S.E., Mitloehner, F.M., 2010. Invited review: Contemporary environmental issues: A review of the dairy industry's role in climate change and air quality and the potential of mitigation through improved production efficiency. *Journal of Dairy Science* 93, 3407-3416. doi: <https://doi.org/10.3168/jds.2009-2719>.

Plomaritou, A., Hanlon, M., Kantas, D., Georgakoudis, K., Dovolou, E., Foskolos, A., 2025. A review of nitrogen use efficiency of dairy replacement heifers: Improving management practices and minimizing nitrogen losses. *Animals* 15, 1031. doi: <https://doi.org/10.3390/ani15071031>.

Powell, J.M., Misselbrook, T.H., Casler, M.D., 2008. Season and bedding impacts on ammonia emissions from tie-stall dairy barns. *Journal of Environmental Quality* 37, 7-15. doi: <https://doi.org/10.2134/jeq2007.0282>.

Russell, J.B., O'Connor, J.D., Fox, D.G., Van Soest, P.J., Sniffen, C.J., 1992. A net carbohydrate and protein system for evaluating cattle diets: I.

- Ruminal fermentation. *Journal of Animal Science* 70, 3551-3561. doi: <https://doi.org/10.2527/1992.70113551x>.
- Russell, W.M.S., Burch, R.L., Hume, C.W., 1959. The principles of humane experimental technique (ed. Russell, W.M.S. and Burch, R.L). Methuen London, UK.
- Schwab, C., Huhtanen, P., Hunt, C., Hvelplund, T., 2005. Nitrogen requirements of cattle. In Nitrogen and phosphorus nutrition of cattle: Reducing the environmental impact of cattle operations (ed. Pfeffer, E., Hristov, A.N.), CAB International, Wallingford, UK, pp. 13-70. <https://doi.org/10.1079/9780851990132.0013>.
- Storm, E., Orskov, E.R., 1983. The nutritive value of rumen micro-organisms in ruminants. 1. Large-scale isolation and chemical composition of rumen micro-organisms. *British Journal of Nutrition* 50, 463-470. doi: <https://doi.org/10.1079/bjn19830114>.
- Su, H., Akins, M.S., Esser, N.M., Ogden, R., Coblenz, W.K., Kalscheur, K.F., Hatfield, R., 2017. Effects of feeding alfalfa stemlage or wheat straw for dietary energy dilution on nutrient intake and digestibility, growth performance, and feeding behavior of holstein dairy heifers. *Journal of Dairy Science* 100, 7106-7115. doi: <https://doi.org/10.3168/jds.2016-12448>.
- Sutton, M.A., van Grinsven, H., Billen, G., Bleeker, A., Bouwman, A.F., Bull, K., Erisman, J.W., Grennfelt, P., Grizzetti, B., Howard, C.M., Oenema, O., Spranger, T., Winiwarter, W., 2011. Summary for policy makers. In *The European Nitrogen Assessment: Sources, effects and policy perspectives* (ed. Bleeker, A., Grizzetti, B., Howard, C.M., Billen, G., van

- Grinsven, H., Erisman, J.W., Sutton, M.A. and Grennfelt, P.), Cambridge University Press, Cambridge, p. xxiv-xxxiv. doi: <https://doi.org/10.1017/CBO9780511976988.002>.
- Tomlinson, D.L., James, R.E., Bethard, G.L., McGilliard, M.L., 1997. Influence of undegradability of protein in the diet on intake, daily gain, feed efficiency, and body composition of Holstein heifers. *Journal of Dairy Science* 80, 943-948. doi: [https://doi.org/10.3168/jds.S0022-0302\(97\)76018-1](https://doi.org/10.3168/jds.S0022-0302(97)76018-1).
- Van Amburgh, M.E., Collao-Saenz, E.A., Higgs, R.J., Ross, D.A., Recktenwald, E.B., Raffrenato, E., Chase, L.E., Overton, T.R., Mills, J.K., Foskolos, A., 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. *Journal of Dairy Science* 98, 6361-6380. doi: <https://doi.org/10.3168/jds.2015-9378>.
- Van Amburgh, M.E., Soberon, F., Meyer, M.J., Molano, R.A., 2019. Symposium review: Integration of postweaning nutrient requirements and supply with composition of growth and mammary development in modern dairy heifers. *Journal of Dairy Science* 102, 3692-3705. doi: <https://doi.org/10.3168/jds.2018-15270>.
- Wathes, D.C., Pollott, G.E., Johnson, K.F., Richardson, H., Cooke, J.S., 2014. Heifer fertility and carry over consequences for life time production in dairy and beef cattle. *Animal* 8, 91-104. doi: <https://doi.org/10.1017/S1751731114000755>.
- Xu, H., Jia, Y., Sun, Z., Su, J., Liu, Q.S., Zhou, Q., Jiang, G., 2022. Environmental pollution, a hidden culprit for health issues. *Eco-*

Environment & Health 1, 31-45. doi:

<https://doi.org/10.1016/j.eehl.2022.04.003>.

Zanton, G.I., Heinrichs, A.J., 2009. Digestion and nitrogen utilization in dairy heifers limit-fed a low or high forage ration at four levels of nitrogen intake. Journal of Dairy Science 92, 2078-2094. doi: <https://doi.org/10.3168/jds.2008-1712>.

Zanton, G.I., Heinrichs, A.J., 2016. Efficiency and rumen responses in younger and older holstein heifers limit-fed diets of differing energy density. Journal of Dairy Science 99, 2825-2836. doi: <https://doi.org/10.3168/jds.2015-10316>.

Appendix I

PUBLICATIONS

➤ **Abstract publications in European Conferences**

1. Foskolos, A., Plomaritou, A., Hanlon, M.E. and Kantas, D. (2023). COWFICIENCY project: a field attempt to increase nitrogen use efficiency of dairy cattle. EAAP 74th Annual meeting, Lyon, France, 26-1 September 2023.
2. Plomaritou, A., Hanlon, M.E., Gatsas, K., Athanasiadis, S., Droumtsekas, K., Kantas, D. and Foskolos, A. (2023). Current status of feed nitrogen use efficiency in dairy replacement heifers in Greece. EAAP 74th Annual meeting, Lyon, France, 26-1 September 2023.
3. Foskolos, A., Plomaritou, A., Hanlon, M.E. and Kantas, D. (2023). Dairy cattle holistic nutritional management for reduced nitrogen pollution. EAAP 74th Annual meeting, Lyon, France, 26-1 September 2023.
4. Plomaritou, A., Hanlon, M.E., Gatsas, K., Pitino, R.G., Tremmas, K., Karatosidi, L., Righi, F., and Foskolos, A. (2021). Evaluation of growth and reproduction performance of replacement dairy heifers. EAAP 72nd Annual meeting, Davos, Switzerland, 30-3 September 2021.

➤ **Abstract publications in National Conferences**

1. Plomaritou, A., Karakosta, A., Fragkakis, K., Karatosidi, L., Gkika, P., Athanasiadis, S., Kantas, D. and Foskolos, A. (2024). Effect of crude protein and rumen degradable protein dietary levels on N balance of dairy replacement heifers. Hellenic Society of Animal Production 38th Annual meeting, Karpenisi, 2-4 October 2024.
2. Plomaritou, A., Hanlon, M. E., Karatosidi, L., Michou, T., Athanasiadis, S., Droumtsekas, K., Kantas, D. and Foskolos A. (2023). Evaluation of dairy replacement heifers management practices in Greece. Hellenic Society of Animal Production 37th Annual meeting, Orestiada, 3-5 October 2023.
3. Plomaritou, A., Hanlon, M. E., Gatsas, K., Athanasiadis, S., Droumtsekas, K. and Foskolos A. (2023). Evaluation of dairy replacement heifers management reared under Greek conditions. 5th Ruminant Workshop, Larissa, 8 December 2023.
4. Plomaritou, A., Hanlon, M. E., Gatsas, K., Tremmas, K., Karatosidi, L., Makridis, L., Kantas, D. and Foskolos A. (2021). Nitrogen use efficiency of the feed of dairy replacement in Greece. Hellenic Society of Animal Production 35th Annual meeting, Athens, 6-7 November 2021.
5. Foskolos, A., Karatosidi, L. and Plomaritou, A. (2020). Growth targets of dairy replacement heifers and Greek management conditions. 2nd Ruminant Workshop, Online, 10 November 2020.

➤ **Publications**

1. Plomaritou A, Hanlon M, Kantas D, Georgakoudis K, Dovolou E, Foskolos A., 2025. A Review of Nitrogen Use Efficiency of Dairy Replacement Heifers: Improving Management Practices and Minimizing Nitrogen Losses. *Animals*, 15(7):1031. doi:<https://doi.org/10.3390/ani15071031>.
2. Foskolos, A., Plomaritou, A., 2023. Guest editorial: Holistic Nutritional Management as a tool to mitigate nitrogen pollution from dairy cattle farming. *The Journal of Agricultural Science*, 161(3):313-317. doi:<https://doi.org/10.1017/S0021859623000254>.

