Preservation of Protein During Harvest and Storage

L. Kung, Jr.[1] and R. E. Muck[2]

[1]University of Delaware, Newark and [2]USDA-ARS, Madison

Objective of This Review

- Review the processes involved that account for the major changes in plant proteins during ensiling
- Describe the factors that can impact these processes
- Discuss methods to reduce proteolysis during ensiling

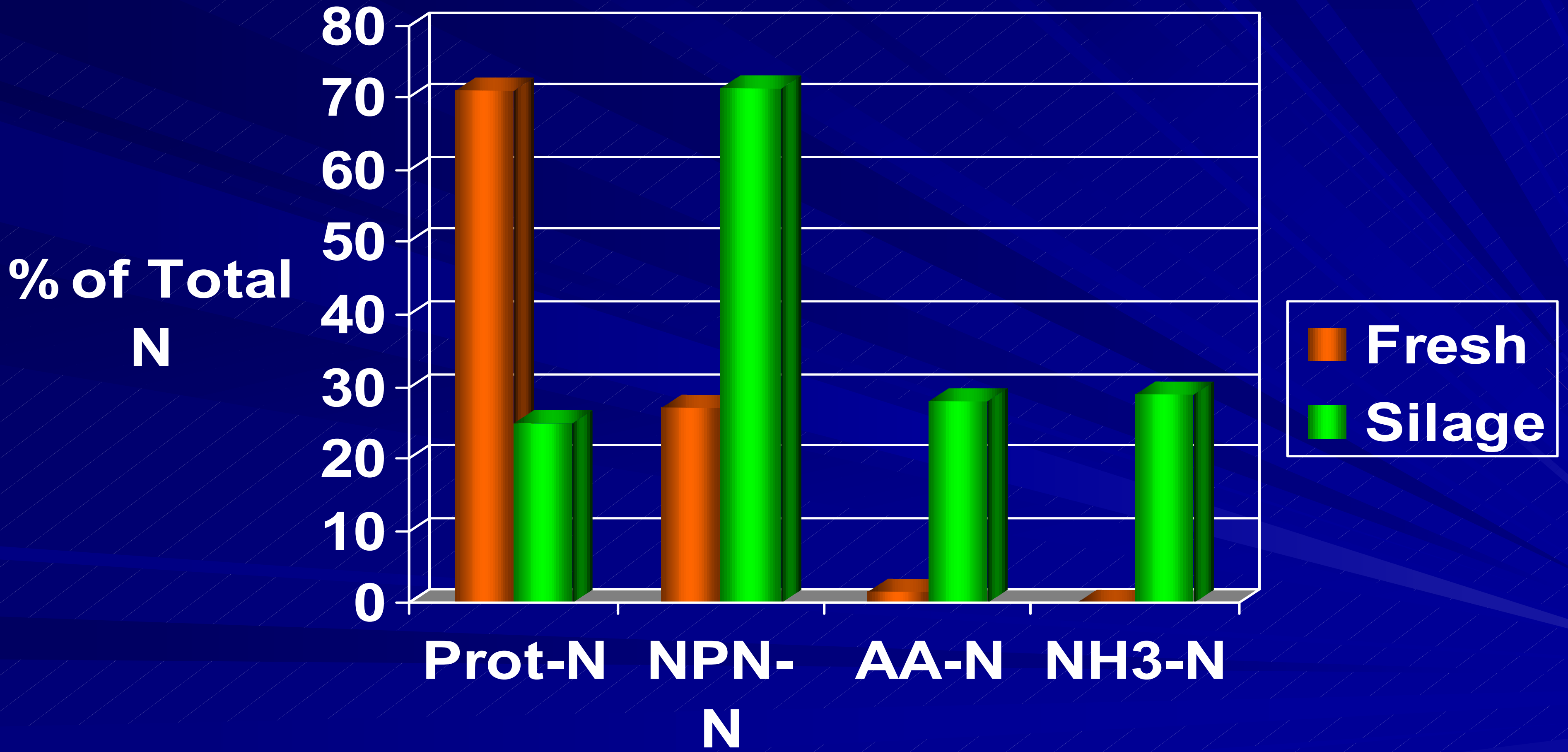
Introduction

- A significant proportion of forage fed to dairy cows is ensiled
- The goal of ensiling is to preserve as much nutrients and energy as possible to feed at a later date
- However, ensiling is an uncontrolled process that can lead to marked degradation of plant protein
- Such losses can approach $100 million annually for alfalfa alone

Distribution of Nitrogen in Perennial Grasses and Legumes

Standing Crop		Post- ensiling
80-95% of CP is true protein a. *Fraction 1 leaf protein – rubisco* *b. Fraction 2 leaf protein* *c. Chloroplast protein*	→	30-40% of CP is true protein
5-20% NPN (peptides, free AA, nitrate and ammonia)	→	60-70% NPN

Changes in N Fractions in Fresh Versus Ensiled Forage



Oshima et al., 1979

Primary Nitrogen Transactions During Ensiling

Two Major Processes Proteins

Plant →

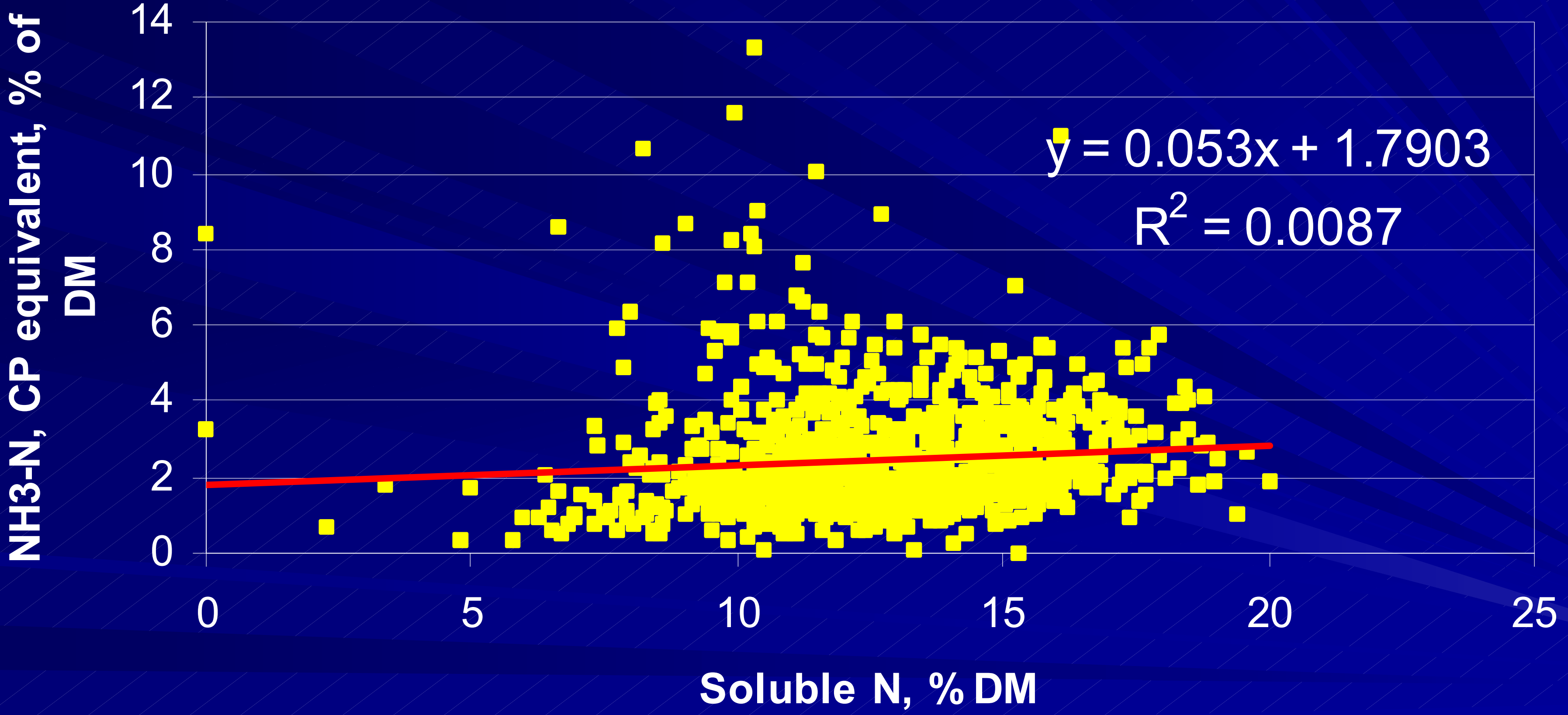
Soluble NPN (AA + Peptides)

Microbial →

Soluble NPN (*NH3-N + AA + Amines)

*NH3-N as a % of total N is usually small ~6-12%

There is No Relationship Between Soluble N and NH3-N in Legume Silages



R. Ward, 2006. CVAS Data set ~ 1300 legume samples

Major Phases of Silage Making

- Phase 1 – Initial Aerobic Phase
- Phase 2 – Primary Fermentation
- Phase 3 – Stable Phase
- Phase 4 – Feed Out Phase

Nitrogen Transactions During Ensiling

- Phase 1 – Initial Aerobic Phase

proteins → **AA**
plant proteases (24-48 h)

AA → NH_3-N
enterobacteria

nitrates → NO, NO_2, NH_3-N
enterobacteria

Nitrogen Transactions During Ensiling

- Phase 2 – Primary Fermentation

  Continuation of Phase 1 factors
  - Plant protease activity declines rapidly
  - Enterobacteria continue until pH < 5.0

  Lactic acid bacteria - some strains
  AA -> NH3-N, amines

Nitrogen Transactions During Ensiling

- Phase 3 – Stable Phase

  proteins → AA

  clostridia

  Only under unfavorable conditions

  AA → NH3-N

  clostridia

- Phase 4 – Feed Out Phase

  Variety of N fractions used by aerobic bacteria and molds

  Maillard reactions with excessive heating

Plant Proteases

- Leaves are always high in protease activity
  - growth and senescence
- pH optimum from 5 to 8 but activity can be found below pH 4 (Heron et al., 1989)
- High temperature optimum: 45 to 55°C
- Mostly carboxyl and thiol proteases
- In freshly grazed forage, may add to total proteolysis in the rumen (Kingston-Smith et al., 2005)

Microbial Activity

- Lactic acid bacteria – limited contribution to proteolysis

  cell envelope proteinases (Prt)

  proteins->oligopeptides

  amino acid decarboxylases

  AA-> amines

- Enterobacteria

  weak proteolytic activity

  amino acid decarboxylase and deaminase

  reduce NO_3

Microbial Activity

- <u>Proteolytic Clostridia</u>

  C. sporogenes, C. bifermentans, C. sphenoides

  Deamination

  Stickland reaction (coupled oxidation and reduction)

Catabolism of AA by Clostridia

- Deamination

  Arg -> citrulline + NH_3

- Decarboxylation

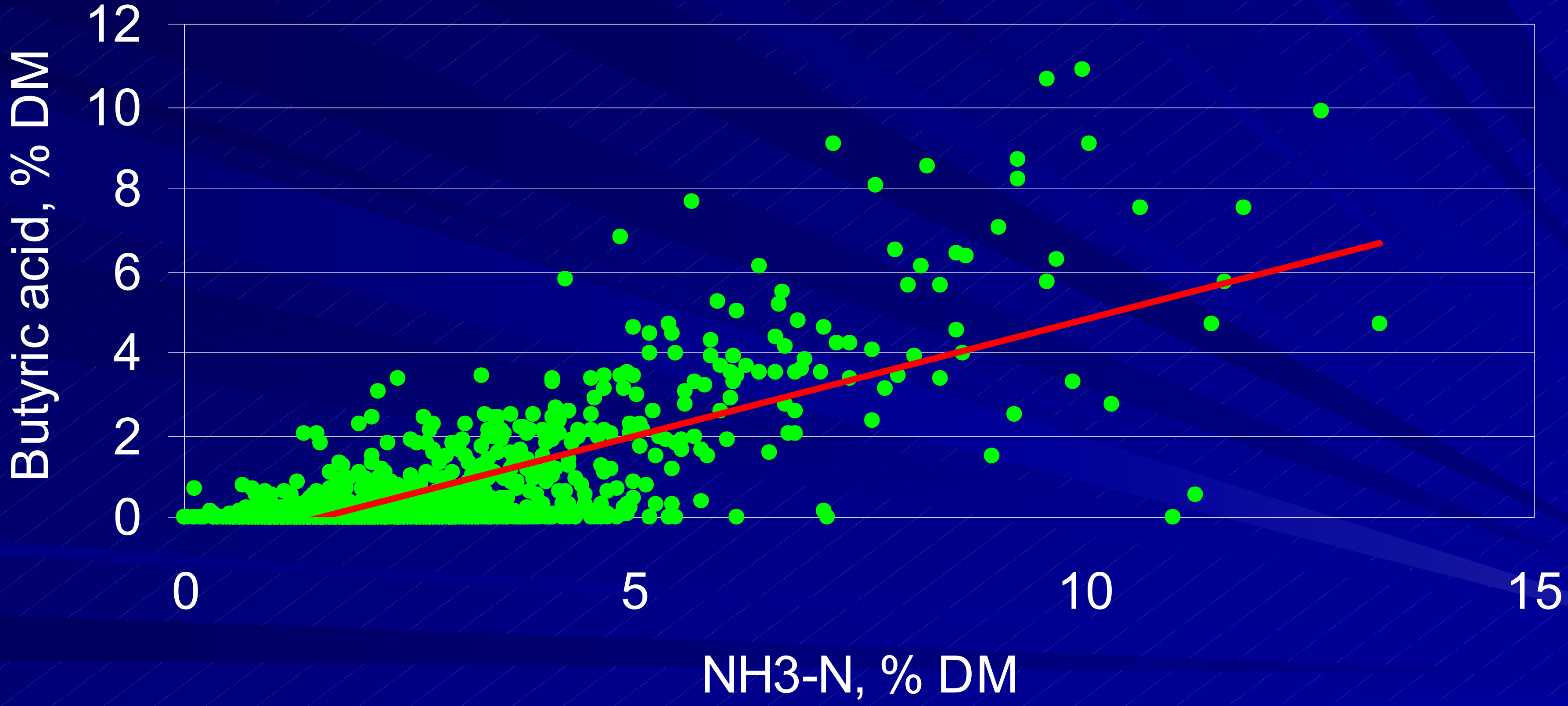
  His -> histamine and CO_2

- Oxidation/Reduction (Stickland Reaction)

  Oxid: Ala + 2 H_20 -> acetic acid + NH_3 + $C0_2$

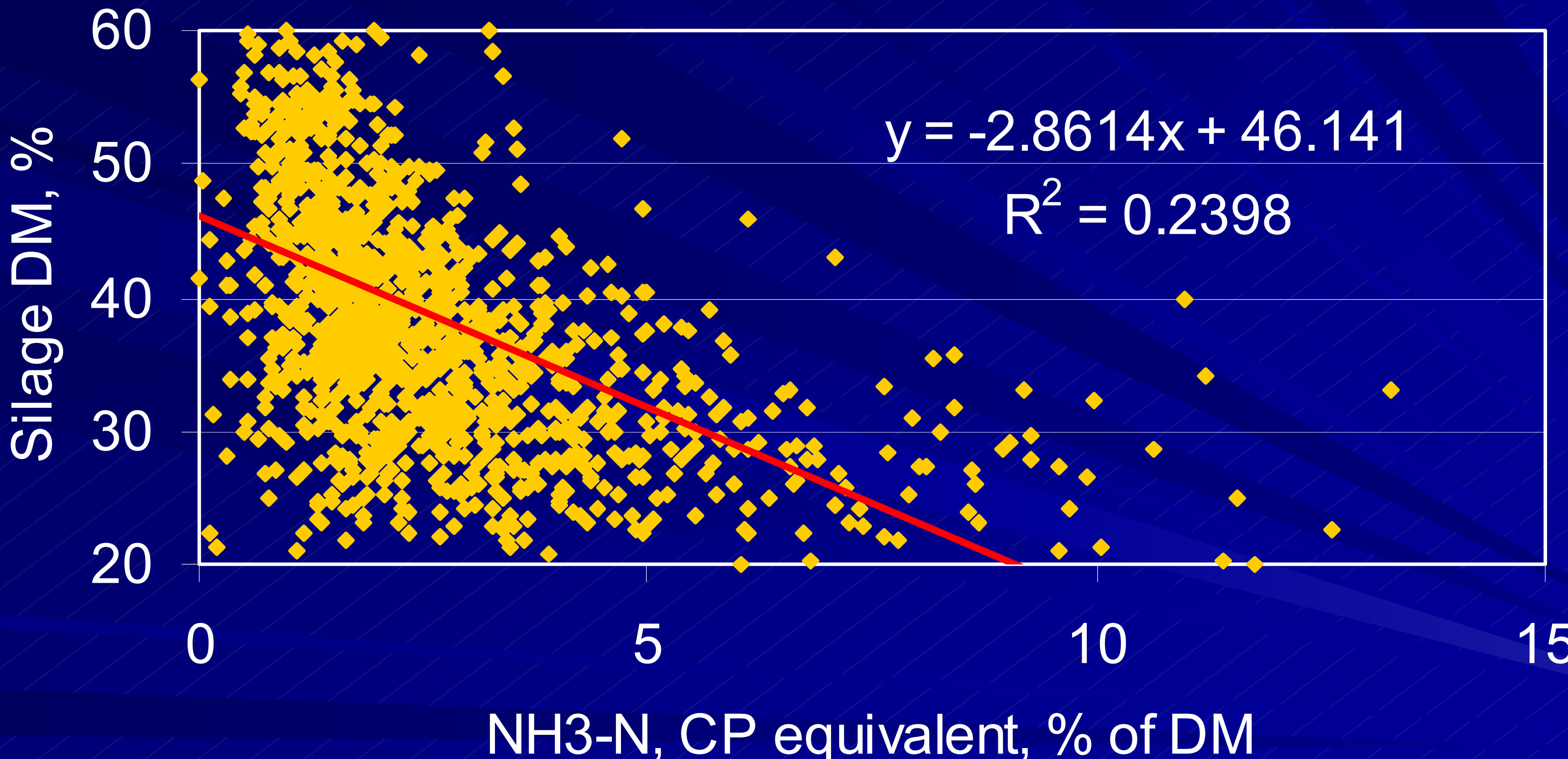
  Red: Gly -> acetic +NH_3

Relationship Between Butyric Acid and Ammonia in Legume Silages



R. Ward, 2006. CVAS Data set ~ 1300 legume samples

NH3-N is Negatively Correlated with Legume Silage DM



R. Ward, 2006. CVAS Data set ~ 1300 legume samples

Example of a Clostridial Grass Silage Where Accumulation of NH3-N Can be Substantial

DM, %	23
CP, %	8
NH3-N, % of CP	54
Butyric acid, %	3.3

Factors Affecting Proteolysis

- Forage type
  legumes > grasses (exc. perennial ryegrass)
  alfalfa > birdsfoot trefoil > red clover

- Temperature
  > with higher temperatures

- pH (rate of decrease, fermentable substrate)
  < with lower pH

Factors Affecting Proteolysis

- Moisture level

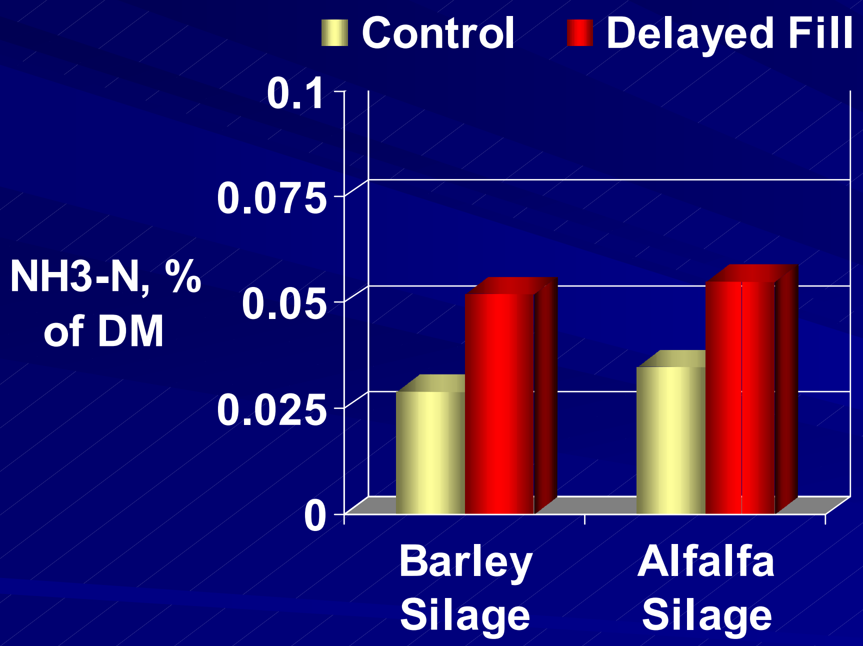
  < rates (but not always amount) as moisture declines

  slow wilting under humid conditions increases proteolysis

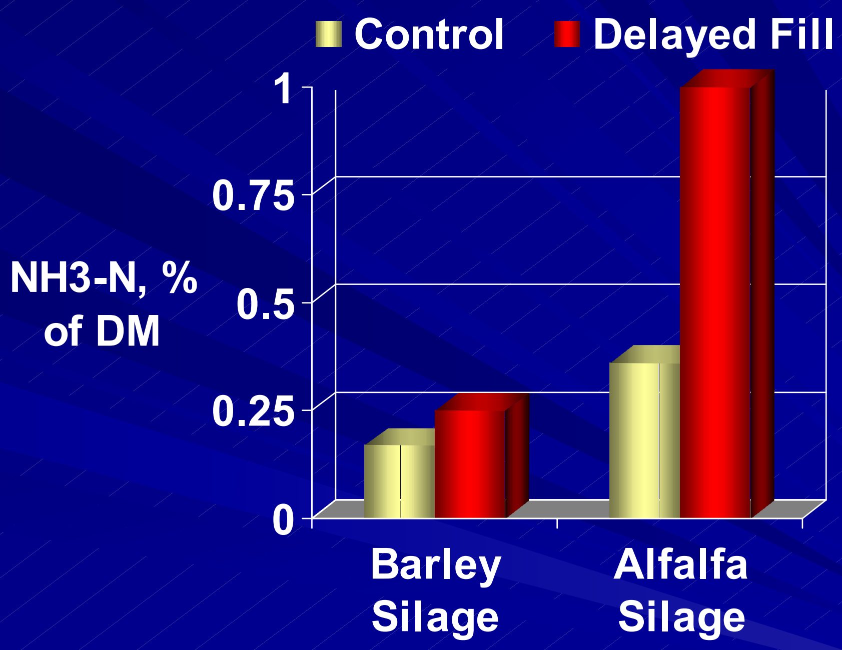
- Oxygen

Effect of Delayed Filling on NH3-N Content of Forages

At Filling



After Ensiling



Probable cause: Plant protease
Enterobacteria

Clostridia

Mills and Kung, 2002; Kung et al., 2005

Methods to Decrease the Extent of Proteolysis During Ensiling

- Restricting fermentation
- Use of silage additives
- Potential to modify plant activity

Methods to Decrease the Extent of Proteolysis During Ensiling

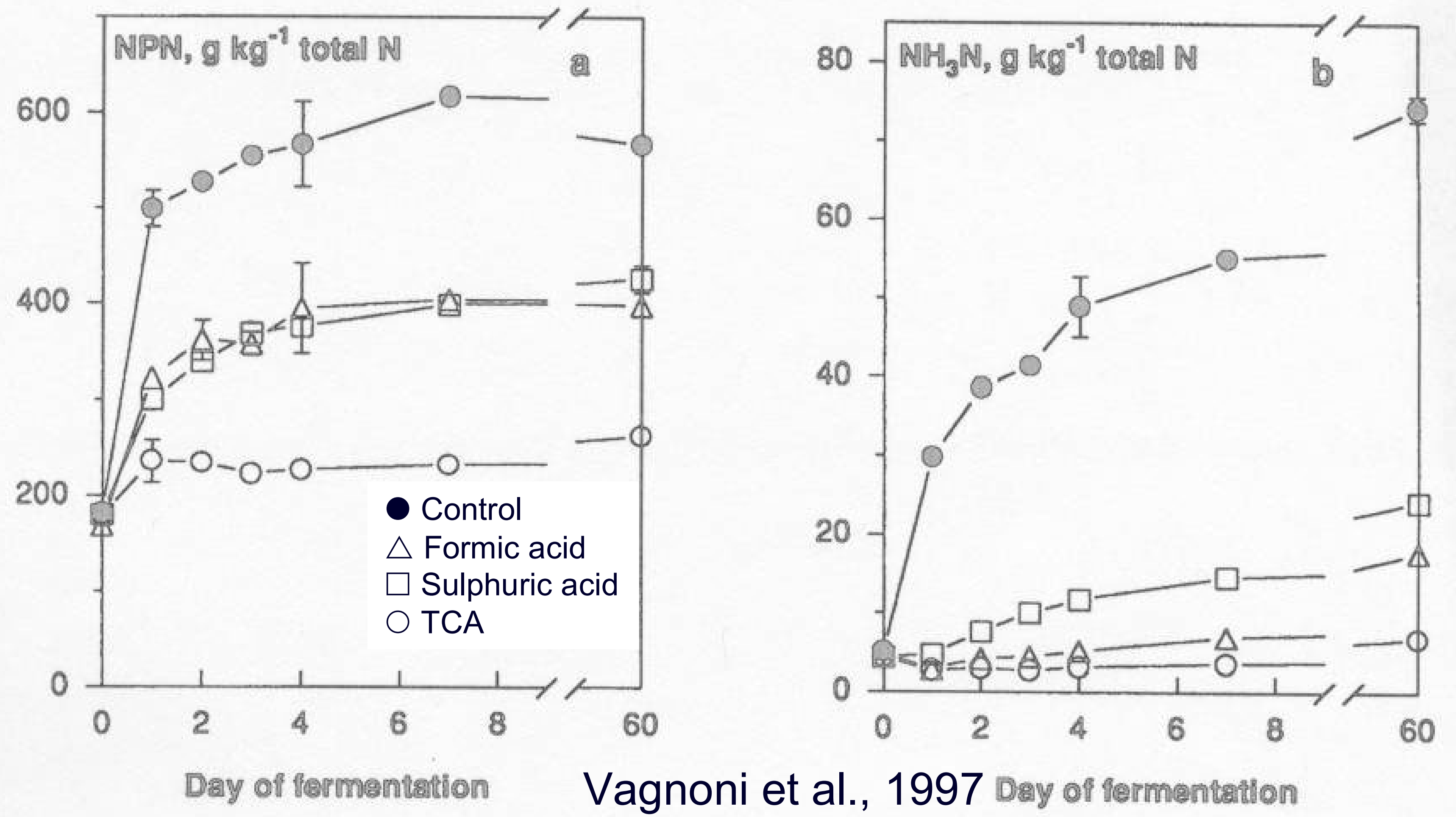
- Restricting fermentation
  - fast wilting to attain 40% DM (wide swath)
  - acidification (not practiced in US)

Methods to Decrease the Extent of Proteolysis During Ensiling

- Additives
  - treatment with acids
  - ammoniation
  - microbial inoculation
  - exogenous protease inhibitors: experimental only

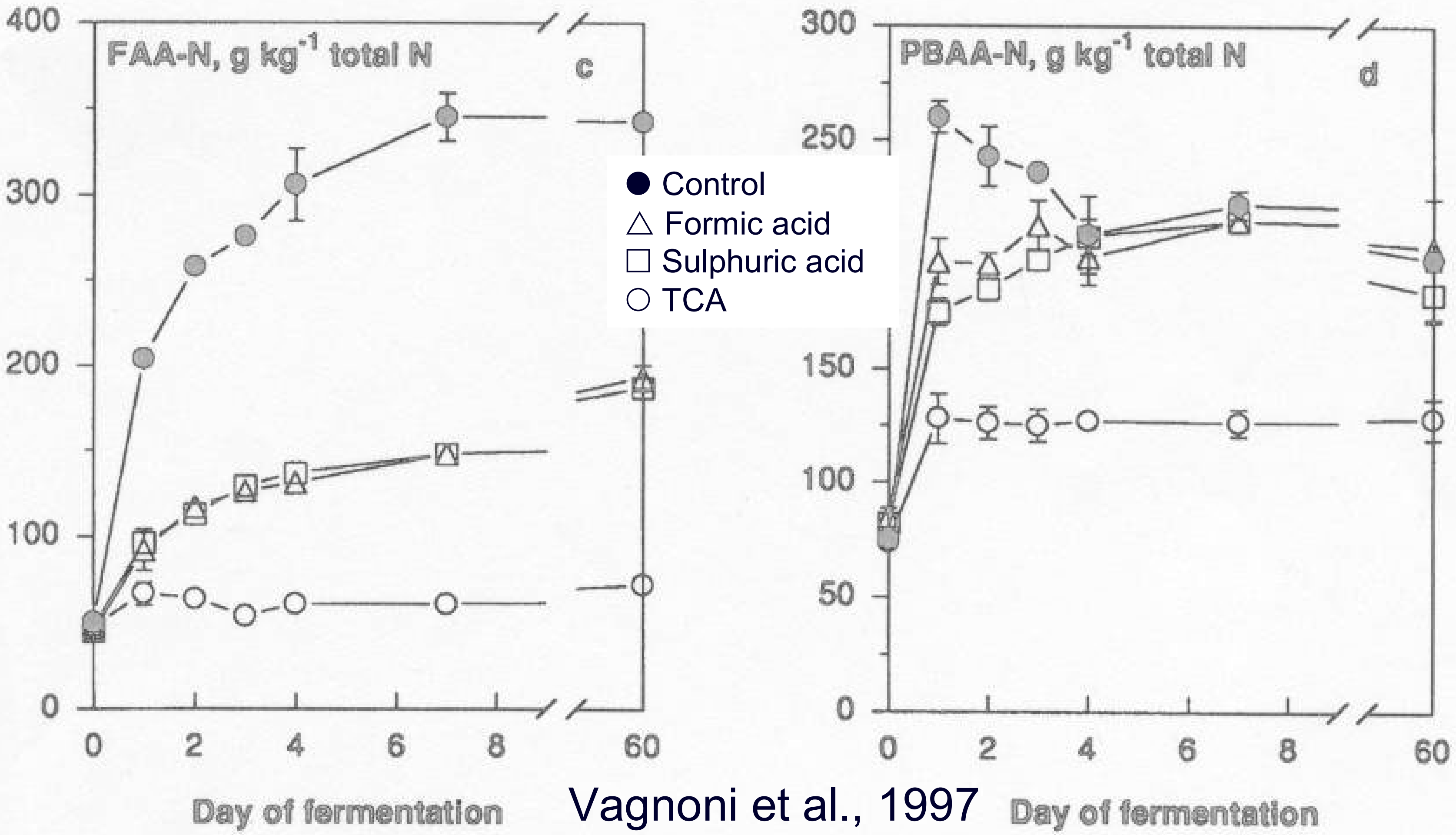
Example of the Effect of Acidification on Proteolysis During Ensiling

Traditional acid treatment slows proteolysis but does not totally destroy protease activity



Vagnoni et al., 1997

Example of Effect of Acidification on Proteolysis During Ensiling



Vagnoni et al., 1997

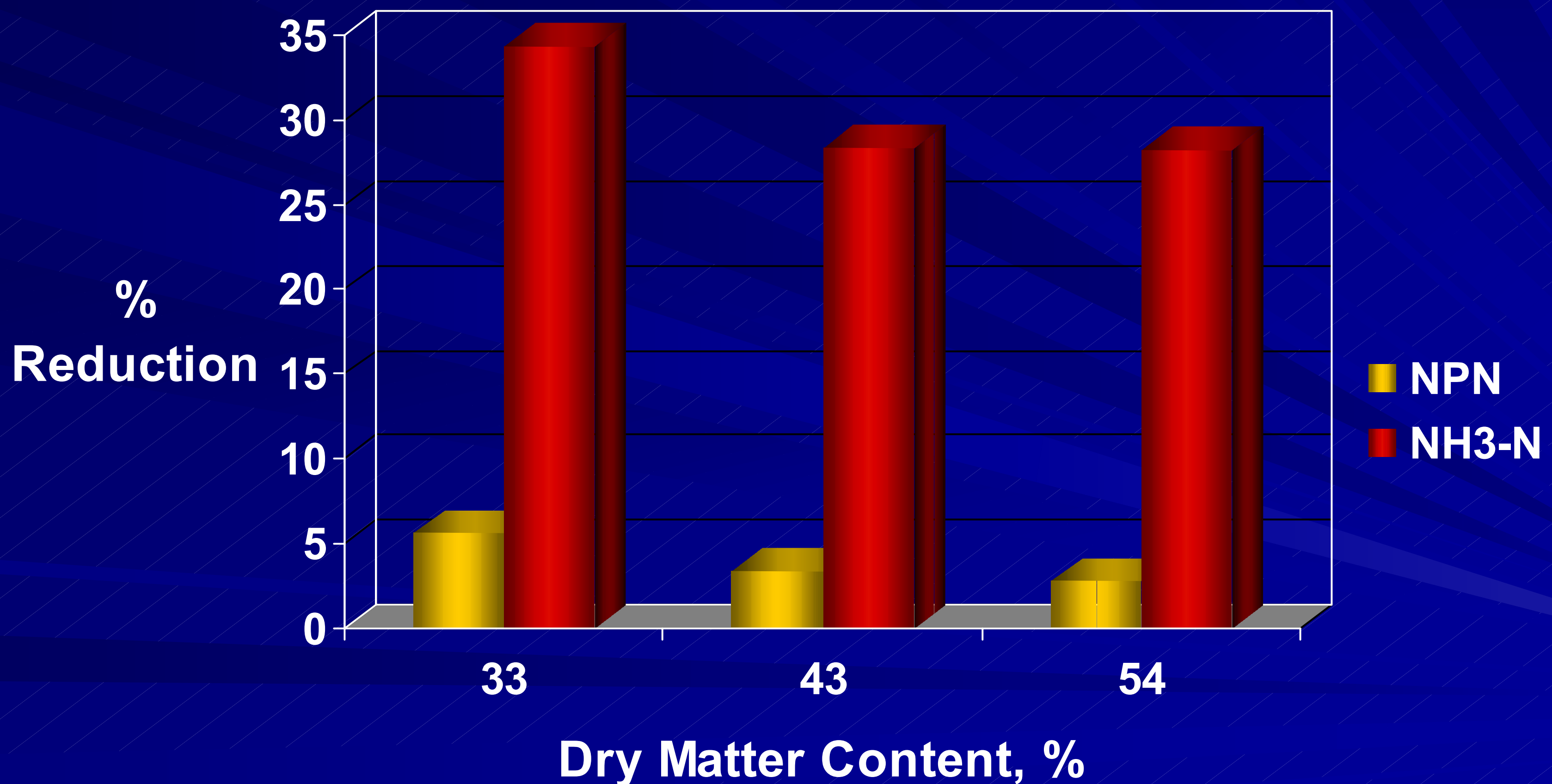
Ammoniation Inhibits Plant Proteolysis

Item	Untreated	Ammoniated
Corn stalklage[1]		
TN, % of DM	0.9	2.0
Insoluble N, % of DM	0.7	1.6
Corn silage[2]		
TN, % of DM	1.4	2.1
True protein N, % of DM	0.7	0.9

[1]Hargreaves et al., 1984

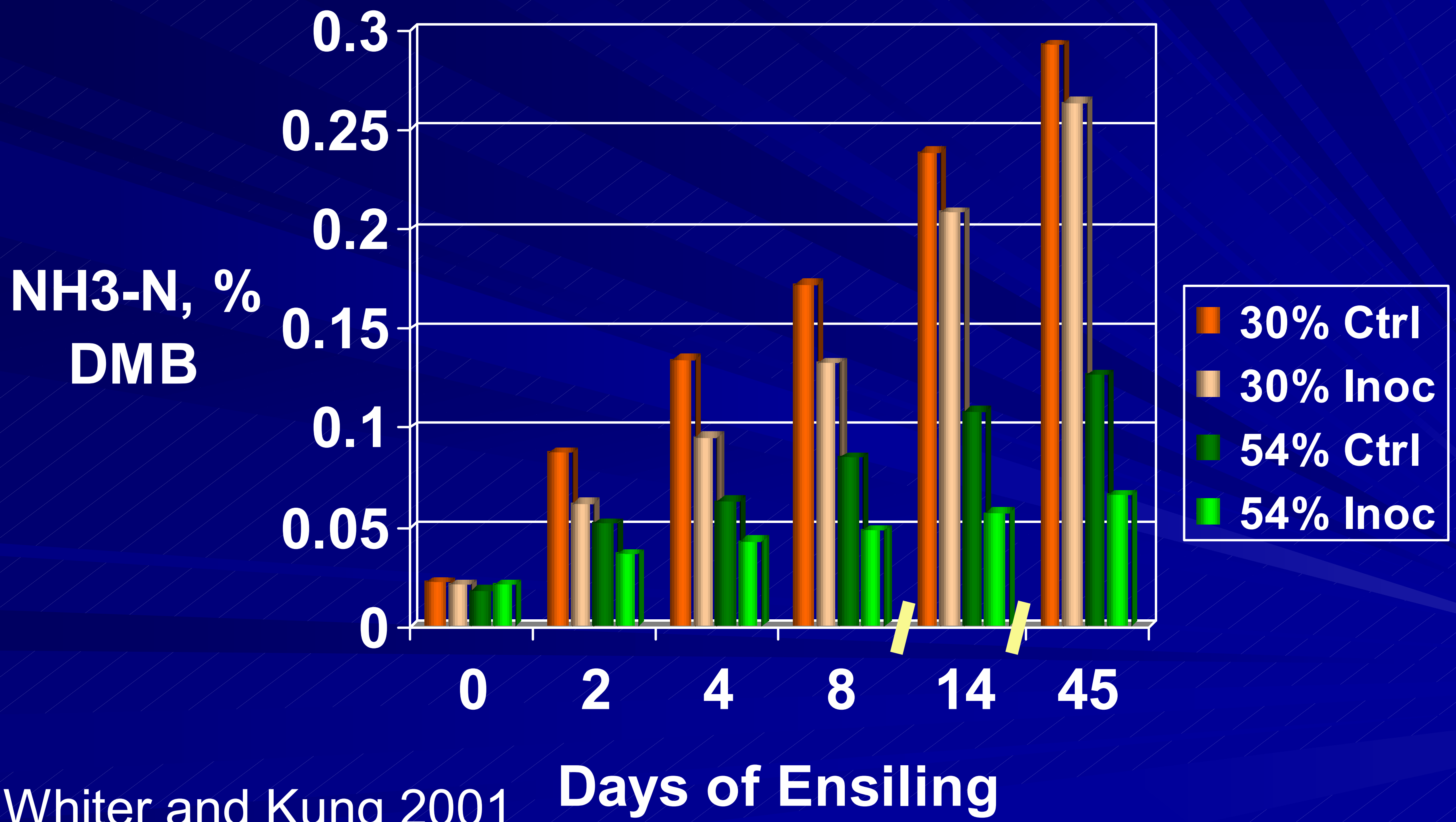
[2]Buchanan-Smith, 1982

Reduction in Soluble NPN and NH3-N In Alfalfa Silage By Inoculation



Jones, et al., 1992

Example of the Effect of DM and Microbial Inoculation on Accumulation of NH3-N in Alfalfa Silage



Whiter and Kung 2001

Effect of Peptidase Inhibitors on Nitrogen Fractions (% of total N) in Perennial Ryegrass Silage

Item	No	E-64[1]	N-em[2]	Cystamine
Soluble N	75	57	57	62
Peptide N	9	7	4	16
Ammonia N	10	9	5	7

[1] 1-trans epoxysuccinyl-leuclamido-(4 guanidino) butane

[2] N ethylmaleimide

Nsereko and Rooke, 1999

Methods to Decrease the Extent of Proteolysis During Ensiling

- Plant modification
  - Polyphenol Oxidase System
  - Low Levels of Tannins

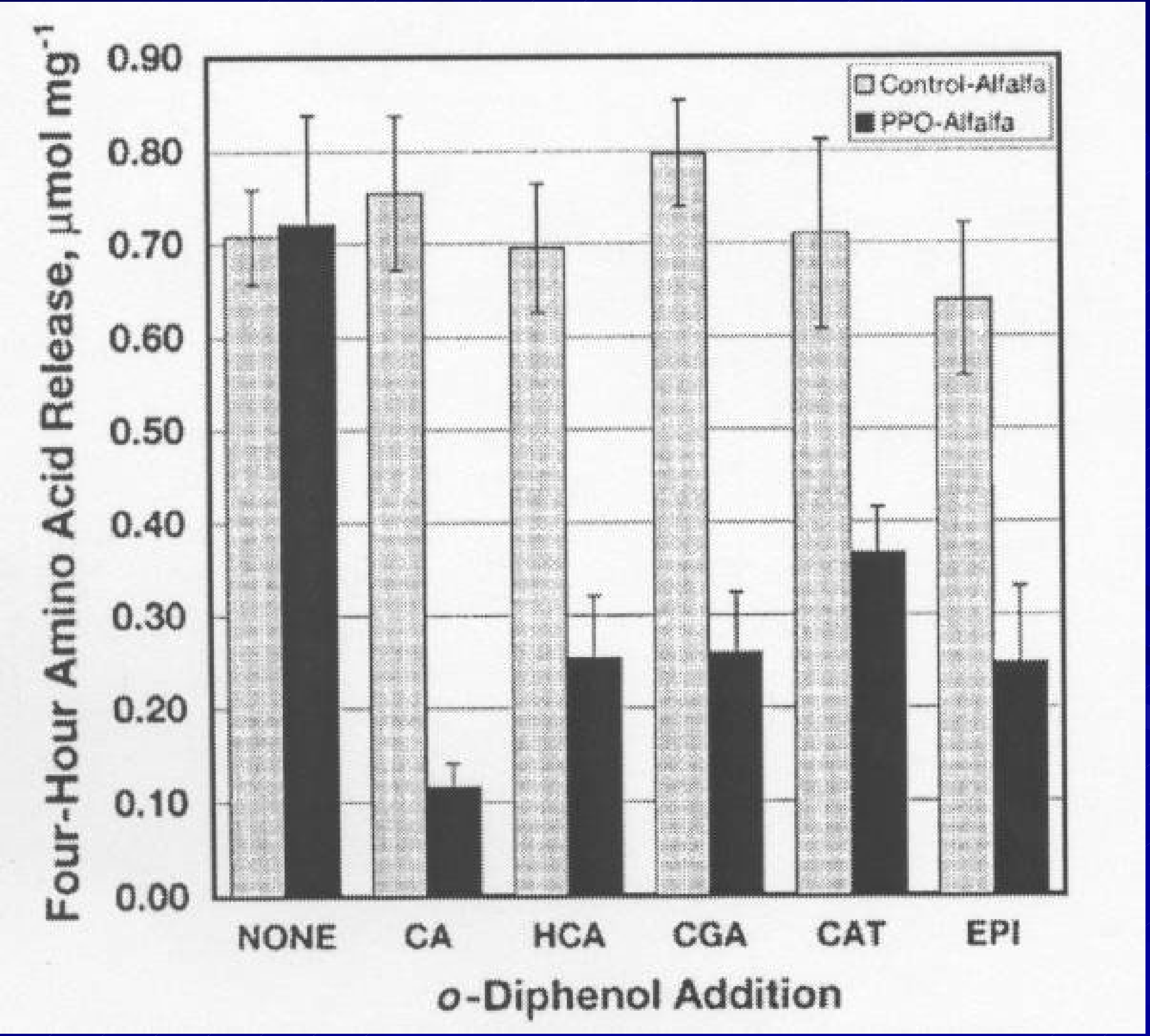
Polyphenol Oxidase (PPO) System

- Red clover has up to 90% less proteolysis than alfalfa during ensiling (Jones et al., 1995, 1996)
  - Alfalfa lacks significant levels of endogenous foliar PPO and diphenols

Polyphenol Oxidase (PPO) System

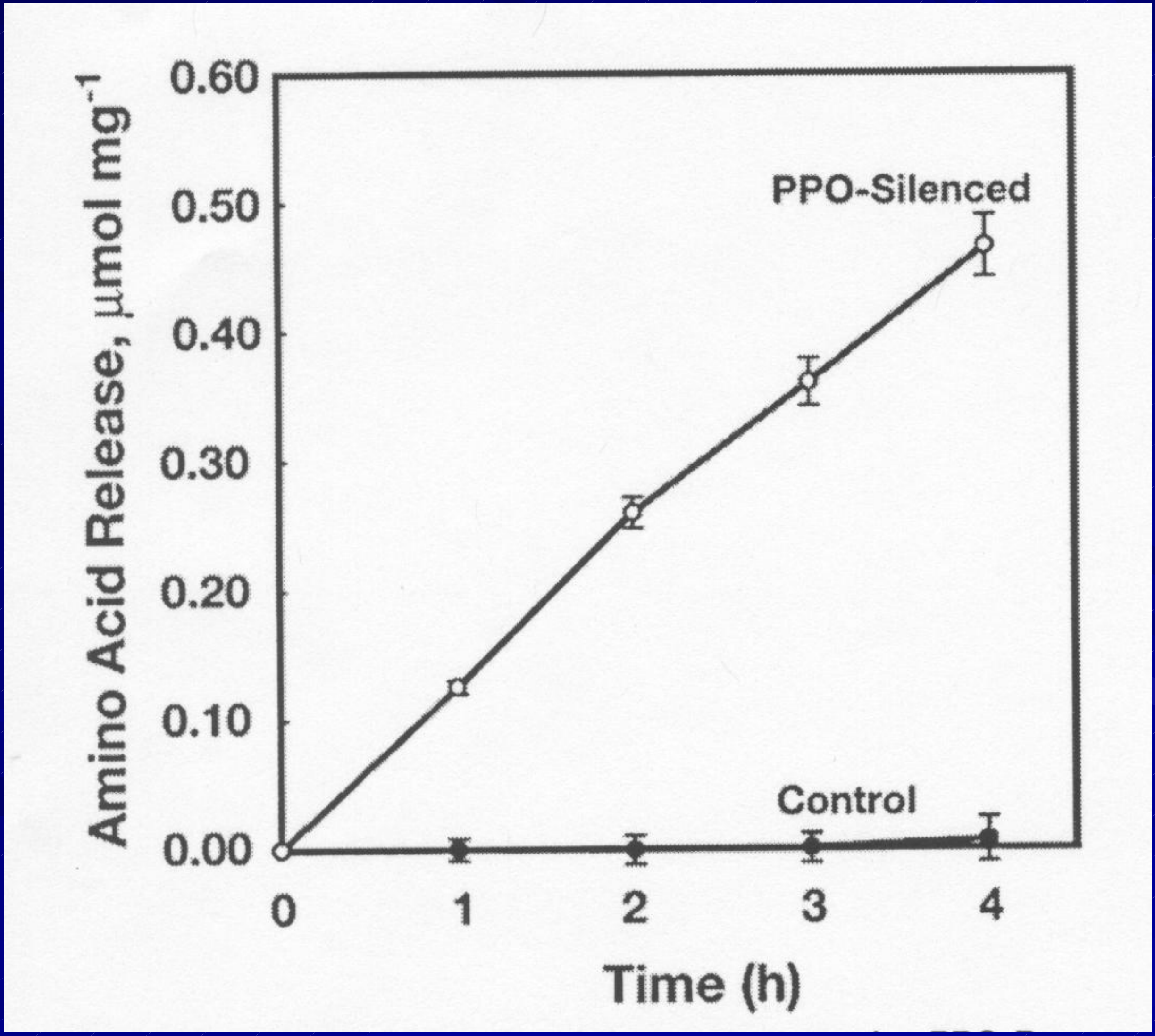
- Pros
  - A single enzyme to be expressed
  - Apply *o*-diphenol substrates when you want the effect - e.g. for ensiling, not hay
- Cons
  - Multiple enzymes need to be expressed if you want both PPO and its substrate
  - Probably not useful for grazing effects

Proteolytic Inhibition Requires *o*-diphenols



Sullivan and Hatfield, 2006

Proteolysis Occurs in PPO-Silenced Red Clover



Sullivan and Hatfield, 2006

Using the PPO System to Inhibit Proteolysis

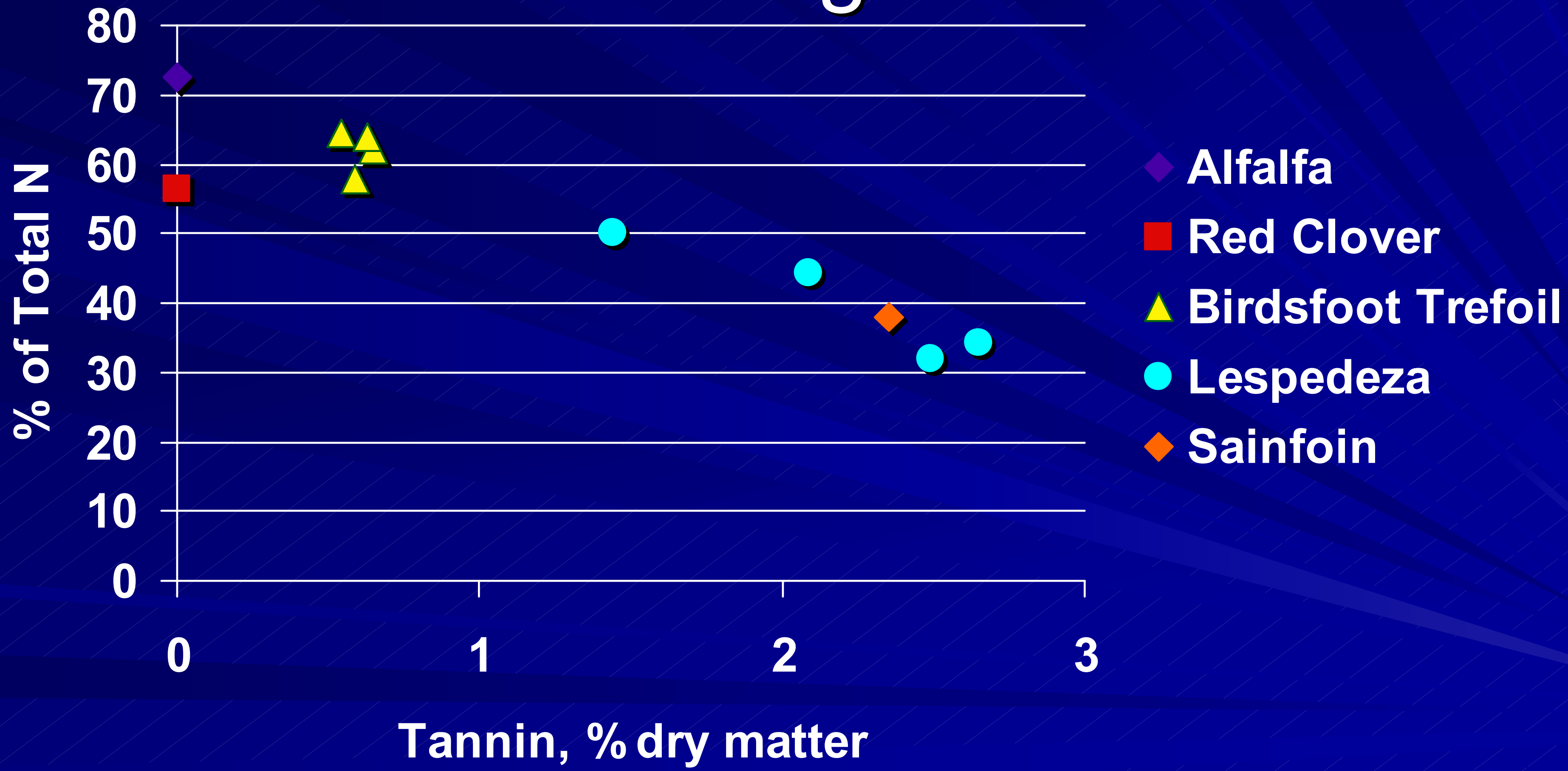
- Supply *o*-diphenols from exogenous sources such as food by products
- Modify secondary plant pathways to produce *o*-diphenols
- Introduce genes to produce PPO

Sullivan and Hatfield, 2006

Tannins

- Condensed tannins bind with plant proteins, bacterial cell surfaces, bacterial enzymes
- Pros
  – Animal effects under all conditions - grazing, hay, silage
- Cons
  – Complicated set of enzymes to express
  – Variability in tannin concentration dependent on growing environment
  – Potential negative animal effects if concentration is too high

Soluble NPN in Silage As Correlated With Tannin in Fresh Forage



Albrecht and Muck, 1991

Breeding Better Species Already with PPO or Tannin

- Red Clover
  - Agronomic issues - persistence, yield
  - Feeding issues - why don’t cows eat this like alfalfa?
- Birdsfoot Trefoil
  - Agronomic issues - persistence, yield
  - Optimize tannin content?

Conclusions

- Proteolysis during ensiling is a major issue with our best forages for milk production
- Breakdown of protein to soluble NPN is largely from plant proteases
- NH3-N and amines are primarily from microbial activity
- Acids like formic could provide substantial reduction in soluble NPN but are unlikely to be adopted

Conclusions

- Ammoniation is effective in reducing proteolysis but is not a good fit for high CP crops like alfalfa and ryegrass.
- Inoculants are beneficial in reducing NH3-N but not the loss of true protein.

Conclusions

- Two mechanisms are found in forage plants for reducing proteolysis: the PPO - *o*-diphenol system in red clover and tannins in many legumes.
- It would be useful to transfer these mechanisms to more productive forage species (alfalfa, ryegrass) to see if they could reduce the loss of true protein during ensiling without adverse effects on their milk producing abilities.