Non-sour fermentations in plant-based foods

PROFERMENT: Solid-state fermentations for protein transformations

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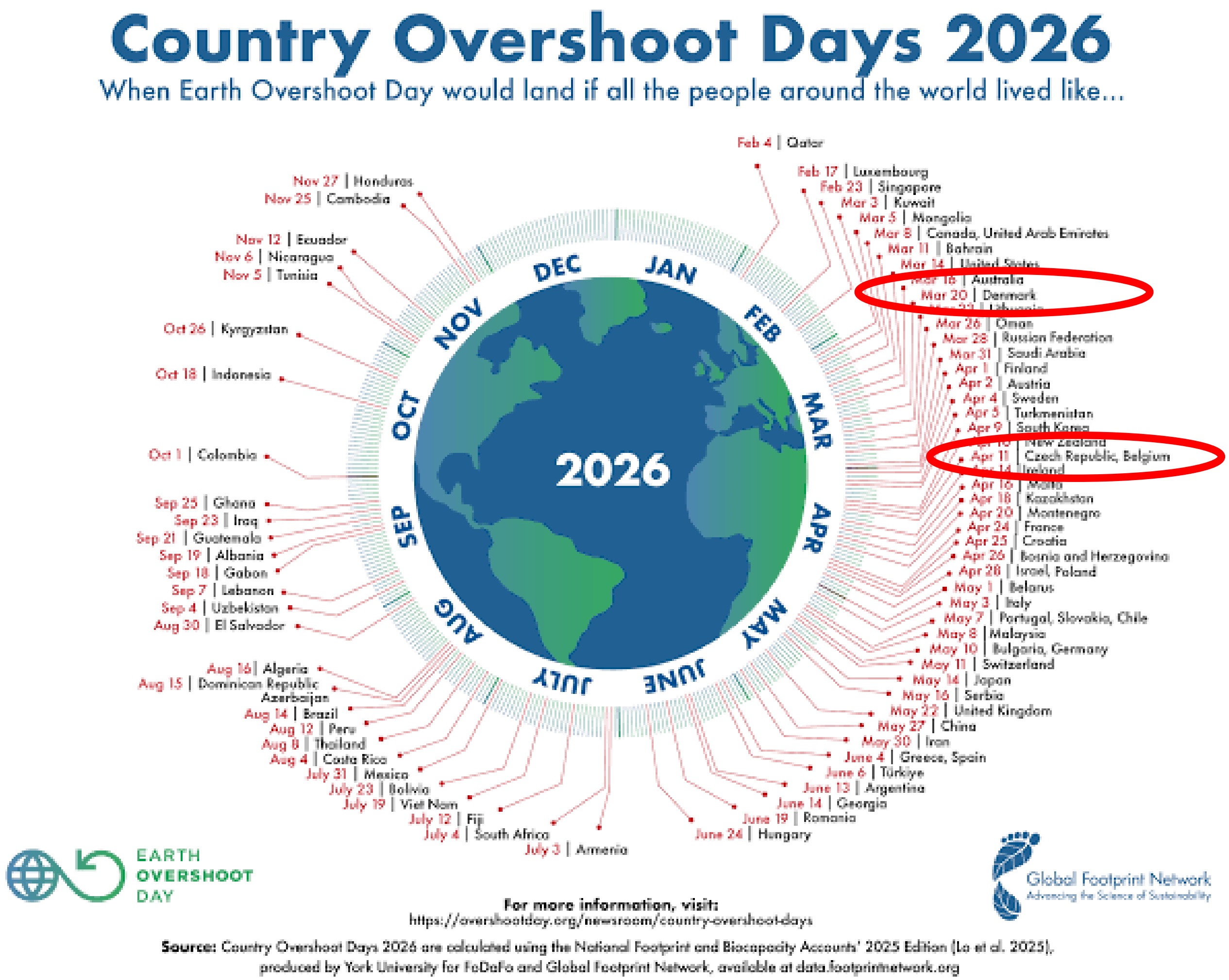


https://overshoot.footprintnetwork.org/newsroom/country-overshoot-days/

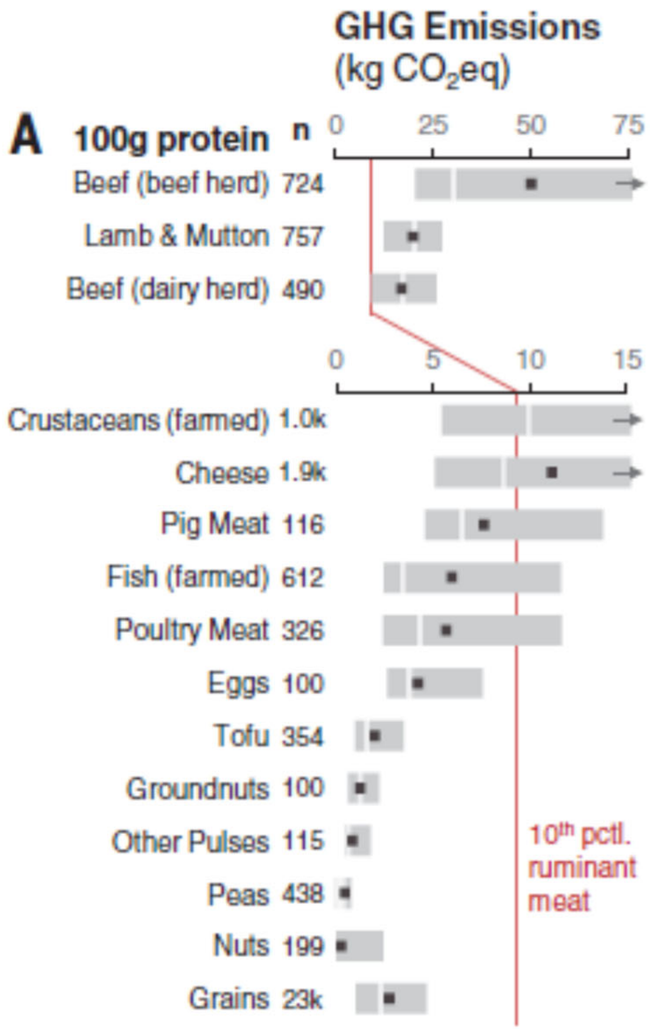
The big sinners

Bad guy

Reducing food's environmental impacts through producers and consumers

J. Poore[1,2]* and T. Nemecek[3]

Poore *et al.*, *Science* **360**, 987–992 (2018)

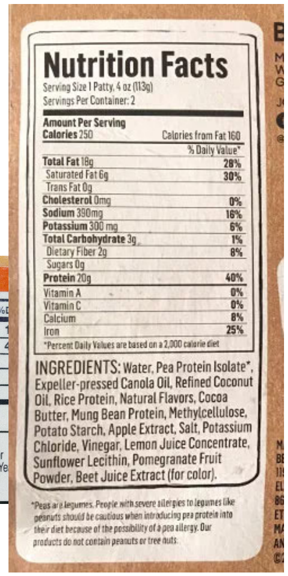


Not that bad guy

Good guys

Current state of the green transition

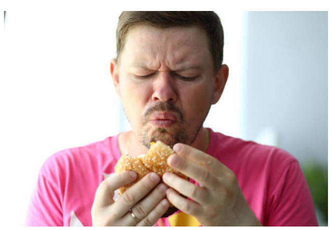
Meat- and dairy analogues from plant-based sources



- ⇨ No (or only little) focus on nutritional quality and health implications
  - ⇨ Trend for 'clean label' and natural products overtaken by transition to plant-based foods
  - ⇨ Malnutrition/non-optimal diet due to anti-nutrients, **lower protein quality (low in some essential amino acids (EAA)**, lack of essential micronutrients (**vitamin B12**)
  - ⇨ Limited knowledge on how extensive processing of plant-proteins influence digestion etc.

Product development

Trial-and-error



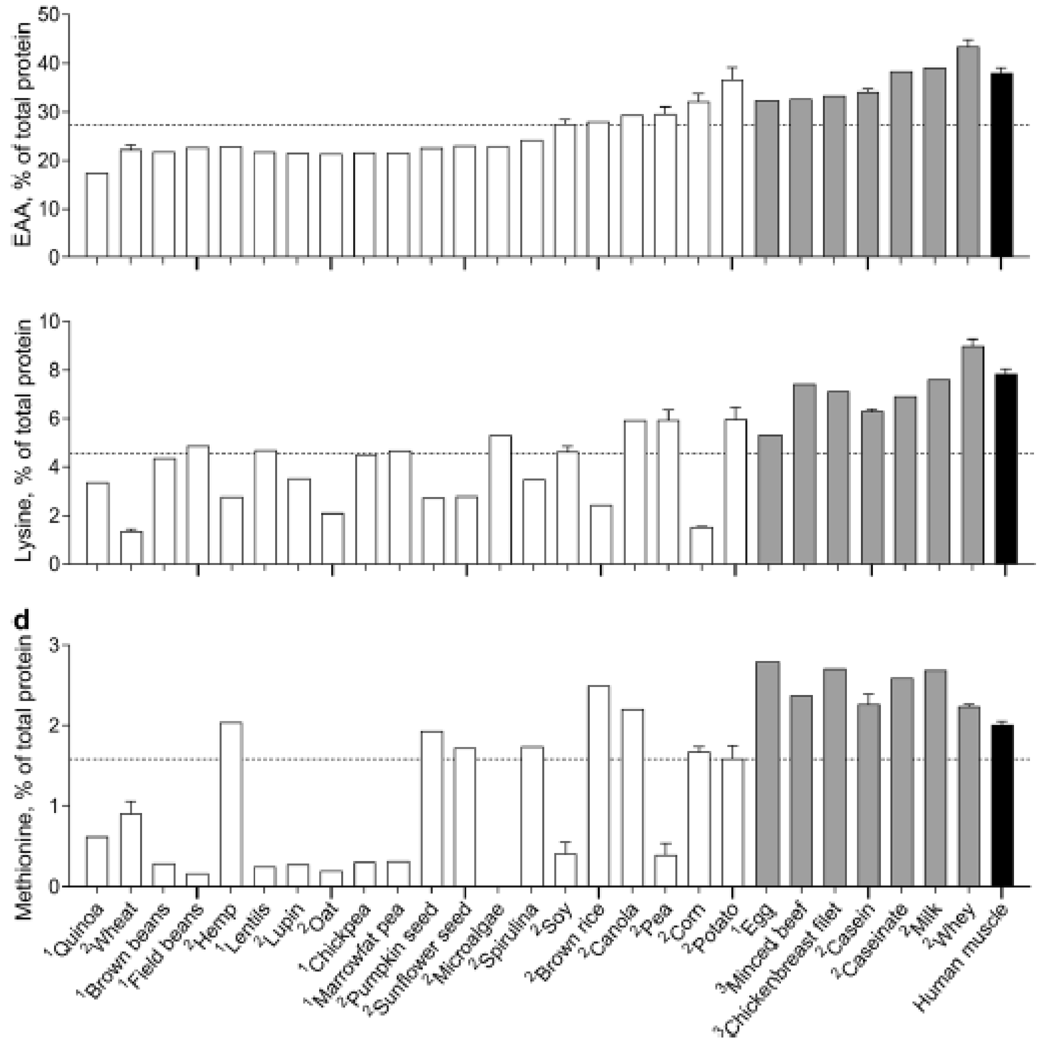
Acceptable quality
No great culinary experience

Not as satisfying food experience as animal-based products -> **slow transition**

Essential amino acids

Plants in general have an amino acid profile that is less optimal than animal sources

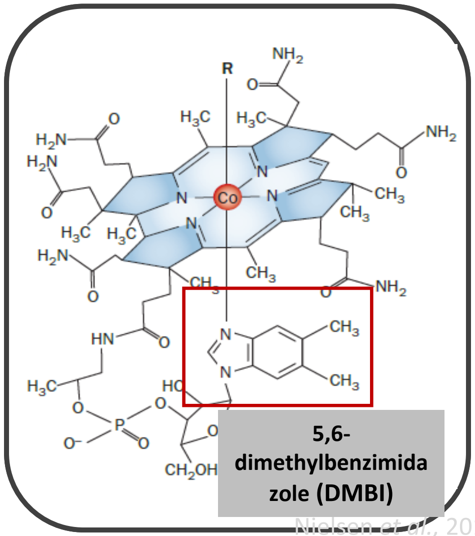
- Especially when it comes to the essential amino acids lysine and methionine



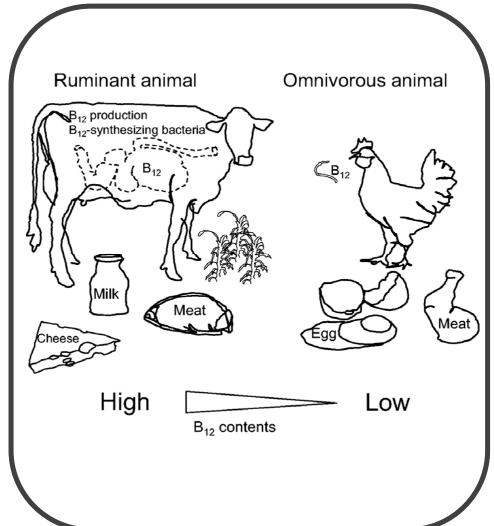
Pinckaers et al. (2021) *Sports Med.* 51 (Suppl 1), S59-S74

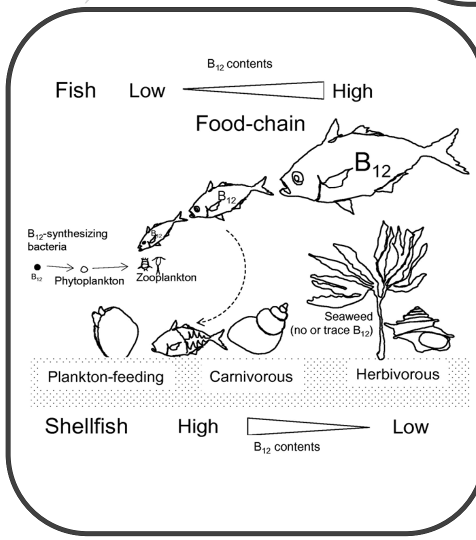
Vitamin B12

- Vitamin B12 is important for formation of red blood cells, the nervous system and DNA/RNA synthesis/repair
- Only produced by a limited number of bacteria and archaea
- Plant-based raw materials do not contain B12

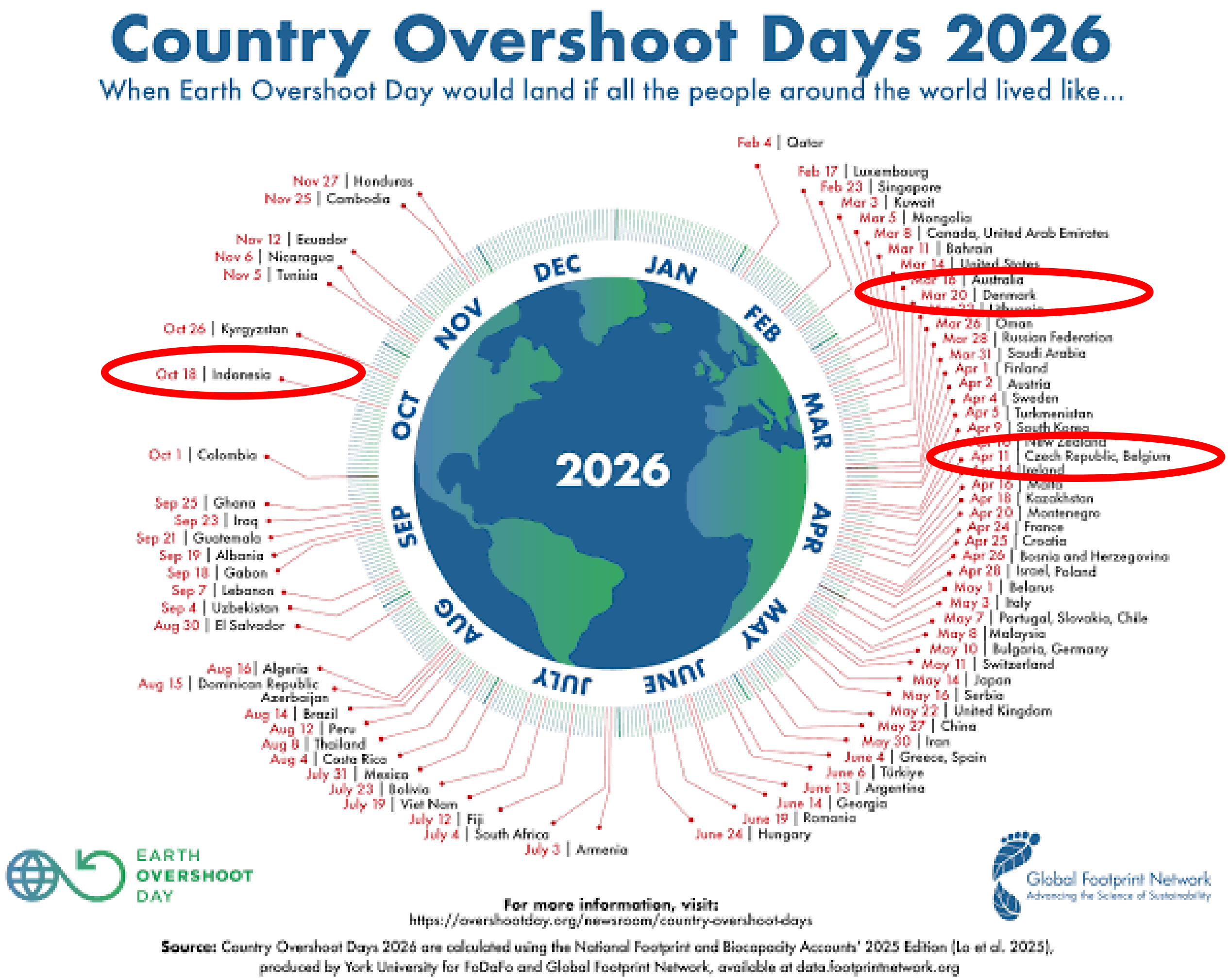


Nielsen et al., 2012





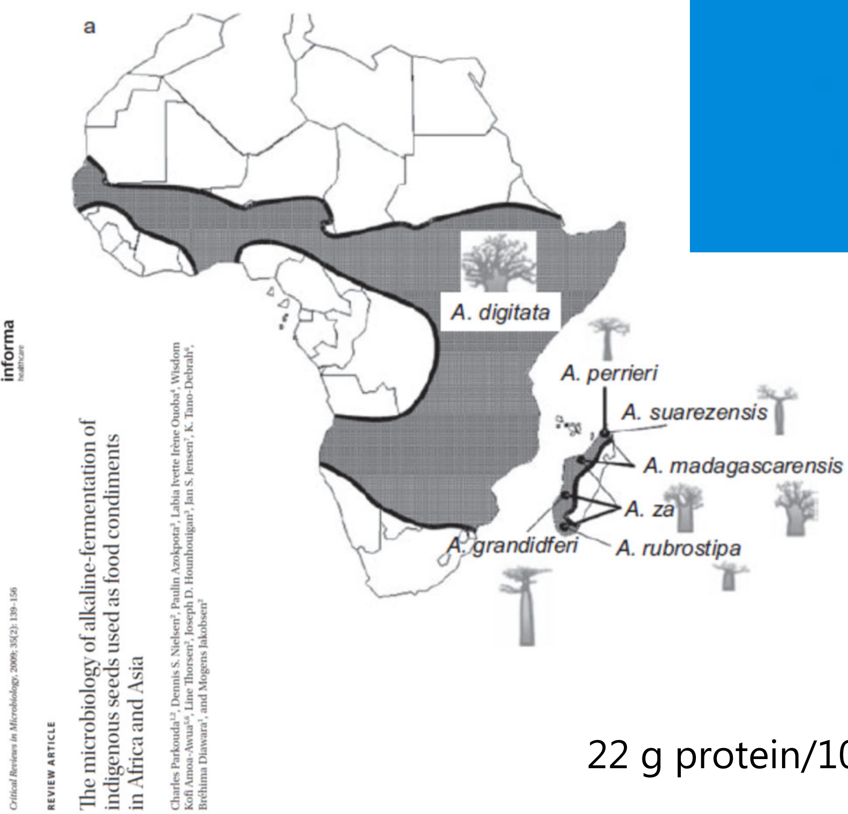
Adapted from Deptula, 2026

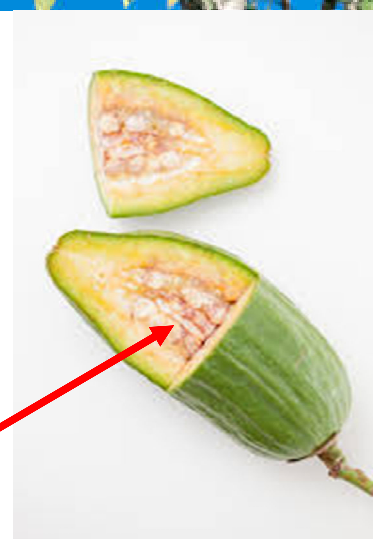


Burkina Faso?

https://overshoot.footprintnetwork.org/newsroom/country-overshoot-days/

Maari – making the inedible edible





22 g protein/100 g

Maari – making the inedible edible

- Maari – a baobab seed based condiment
- Spontanous fermentation

International Journal of Food Microbiology 142 (2010) 292–301

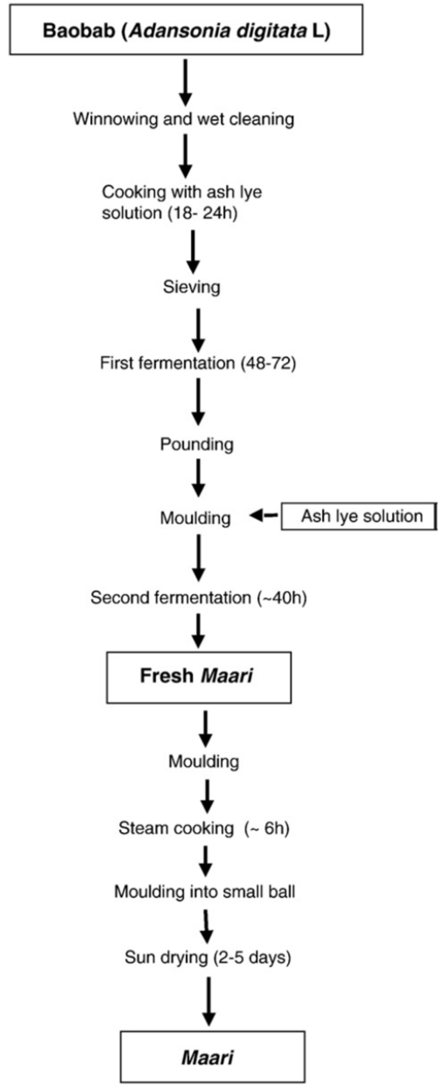
Contents lists available at ScienceDirect

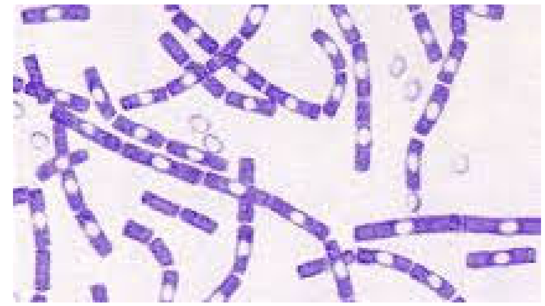
International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Microorganisms associated with Maari, a Baobab seed fermented product

Charles Parkouda [a,b], Line Thorsen [b], Clarisse S. Compaoré [a], Dennis S. Nielsen [b,*], Kwaku Tano-Debrah [c], Jan S. Jensen [d], Bréhima Diawara [a], Mogens Jakobsen [b]





Maari – a flavour enhancer (and a source of protein)

Table 2
Amino Acid Profiles (nmol/mg dry sample) of baobab Raw Seeds, Fermenting baobab Seeds Taken at Different Fermentation Times, and Maari (ready to use) from Different areas (Gorgadji, Mansila, and Ouagadougou).

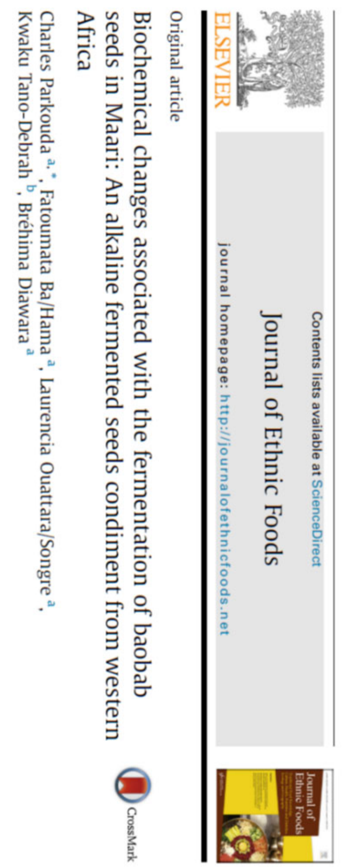
	Valine	Leucine	Isoleucine	Threonine	Methionine	Phenylalanine	Lysine
Raw seeds	0.40 ± 0.06	0.41 ± 0.06	0.95 ± 0.12	0.18 ± 0.06	0.03 ± 0.00	0.19 ± 0.04	0.03 ± 0.00
24 h	0.51 ± 0.72	8.36 ± 0.15	0.86 ± 1.22	ND	ND	0.15 ± 0.02	ND
36 h	1.98 ± 0.24	2.86 ± 0.45	4.10 ± 0.47	0.20 ± 0.05	0.10 ± 0.03	1.11 ± 0.17	0.06 ± 0.01
48 h	4.43 ± 0.21	6.58 ± 0.62	8.21 ± 0.12	0.42 ± 0.6	0.77 ± 0.09	3.71 ± 0.3	0.41 ± 0.05
60 h	15.92 ± 0.93	14.53 ± 1	28.57 ± 1.86	1.05 ± 0.17	ND	8.92 ± 0.58	2.71 ± 0.14
Maari 1	7.07 ± 0.16	6.63 ± 0.27	10.95 ± 0.35	0.32 ± 0.09	0.79 ± 1.53	2.07 ± 0.09	0.63 ± 0.03
Maari 2	16.12 ± 4.7	18.11 ± 3.01	9.85 ± 2.05	9.16 ± 2.44	5.16 ± 1.5	7.12 ± 2.2	10.84 ± 2.4
Maari 3	4.73 ± 0.00	4.96 ± 0.11	7.79 ± 0.12	1.05 ± 0.01	0.89 ± 0.04	1.98 ± 0.00	0.56 ± 0.00
Maari 4	5.14 ± 0.32	5.19 ± 0.64	8.19 ± 0.96	ND	0.42 ± 0.08	1.53 ± 0.24	0.44 ± 0.08

Values reported represent the average of three determinations ± standard deviation.
Maari 1, final product; Maari 2, final product (ready to use) from Gorgadji; Maari 3, final product (ready to use) from Mansila; Maari 4, final product (ready to use) from Ouagadougou; ND, not detected.

Table 2 *(Continued)*

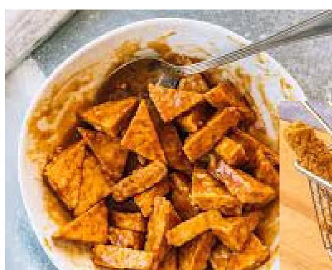
Tryptophan	Histidine	Alanine	Aspartic acid	Aspargine	Glutamic acid	Glycine	Proline	Serine	Total FAA
0.02 ± 0.00	ND	8.83 ± 1.33	1.63 ± 0.21	0.52 ± 0.18	1.94 ± 0.04	0.58 ± 0.11	0.32 ± 0.04	ND	16.03
ND	ND	2.61 ± 0.57	0.43 ± 0.6	ND	1.91 ± 0.70	0.38 ± 0.53	0.10 ± 0.1	ND	15.31
ND	ND	3.40 ± 0.48	1.78 ± 0.17	ND	10.51 ± 2.03	0.62 ± 0.08	1.20 ± 0.19	ND	27.94
ND	ND	4.97 ± 0.62	2.65 ± 0.23	0.67 ± 0.13	15.73 ± 2.09	1.09 ± 0.05	1.24 ± 0.17	ND	50.90
0.75 ± 0.05	0.50 ± 0.06	28.10 ± 2.7	2.10 ± 0.29	ND	1.61 ± 0.17	7.50 ± 0.7	0.99 ± 0.12	ND	113.24
0.09 ± 0.01	ND	9.95 ± 0.35	1.14 ± 0.1	ND	34.04 ± 1.53	1.39 ± 0.08	3.16 ± 0.08	ND	78.22
ND	ND	29.64 ± 11.2	26.18 ± 5.9	ND	31.07 ± 5.29	14.26 ± 4.3	9.60 ± 3.6	14.11 ± 5.8	201.26
0.07 ± 0.00	ND	5.56 ± 1.21	2.41 ± 0.03	0.80 ± 0.02	17.42 ± 0.63	3.10 ± 0.38	0.66 ± 0.01	ND	51.98
ND	ND	8.21 ± 0.54	0.96 ± 0.15	ND	21.80 ± 3.54	1.10 ± 0.12	3.36 ± 0.35	ND	56.35

=> Proteolysis and *de novo* synthesis



Tempeh

- A traditional Indonesian stable food
  - Traditionally soya-based
- *Rhizopus* based
  - *R. microspores* var. *oligosporus*
    - Good producer of e.g. vitamin B2, nicotinic acid, nicotinamide, and vitamin B6
    - (*R. oryzae, R. microspores* var. *chinensis...*)
  - Rapidly grows a strong mycelium
  - Rather taste neutral (nutty flavour)



Dehydrated raw bean, legume, or grain

↓

Soak in clean water for 6-24 hours until hydrated and tender

↓

Dehull manually by hand or mechanically, skim hulls from water (optional)

↓

Boil for 20-45 minutes, skim remaining hulls from water

↓

Dry beans with clean towel or air dry

↓

Cool down to 25°C

↓

Inoculate with *Rhizopus* spp. spores (approx. 10^4 CFU/g), mix thoroughly

↓

Pack into choice of leaf or perforated bag, or tray with semi-aerobic atmosphere

↓

Incubate at 27-30°C for 30-48 hours

↓

Harvest, pasteurize, dry, freeze, or process in other ways

Compr Rev Food Sci Food Saf. 2021;20:1717–1767.

The PROFERMENT vision, hypothesis and goal

- PROFERMENT: Solid-state fermentations for protein transformations and palatability of plant-based foods
  - Novo Nordisk Foundation funded Challenge project (7.7 MEuro, 2022-2027)
- Our vision: Build the scientific foundation for future development of **proteinaceous, plant-based** foods as **clean-label** meat alternatives via **solid-state fermentation**
- We hypothesise: Via fungal and *Bacillus* solid-state **plant co-fermentations**
  - ✓ protein quality and accessibility can be improved, and
  - ✓ desired structures and flavour precursors generated
- We aim to: Understand how *Bacillus, Aspergillus* and *Rhizopus* microbial interactions in plant-matrices safely will
  - ✓ Enhance the **EAA content** (with particular focus on lysine and methionine)
  - ✓ Increase **protein bioavailability**
  - ✓ Generate desirable **texture/structures** and savoury **flavours**

The PROFERMENT work horses

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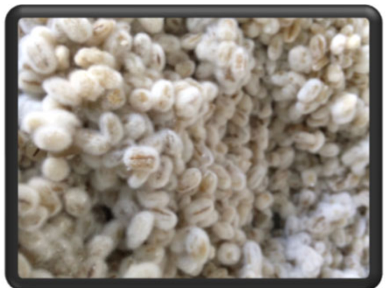
Oat. 25.8 Mt
≈75% as feed
12-24% protein
Gluten-free

Aspergillus

- "Koji-moulds"
- Wide **CAZymes diversity**
- Very good at breaking down cellulose etc.
- Amino acid transformations



Yellow pea. 12.8 Mt
+50% as feed
21-33% protein
Nitrogen-fixating

Bacillus

- **GRAS** (Generally Recognised As Safe)/QPS species
- Multiple **proteolytic and carbohydrate enzymes**
- **Fungal attachment**
- **Lysine and methionine production**
- Secondary metabolites (e.g. B1**), flavour precursors**

Co-culture synergisms for:

- **Synergistic growth/interactions**
- **Matrix protein mobilization and transformation** (protein quality and accessibility)
- **Palatability** (flavour and texture)

Rhizopus

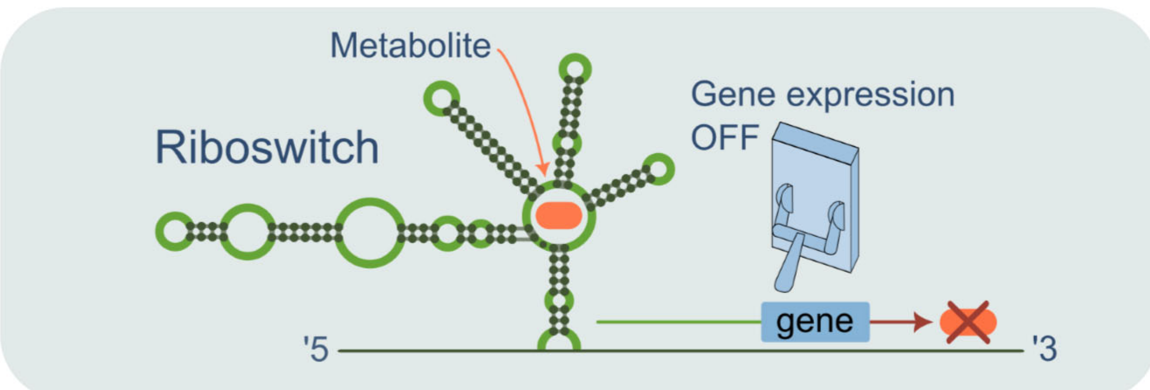
- Focus on **GRAS species**
- Rapidly build strong **hyphae network**
- **Structure, tensile strength and elasticity**
- Wide **enzymatic activity**

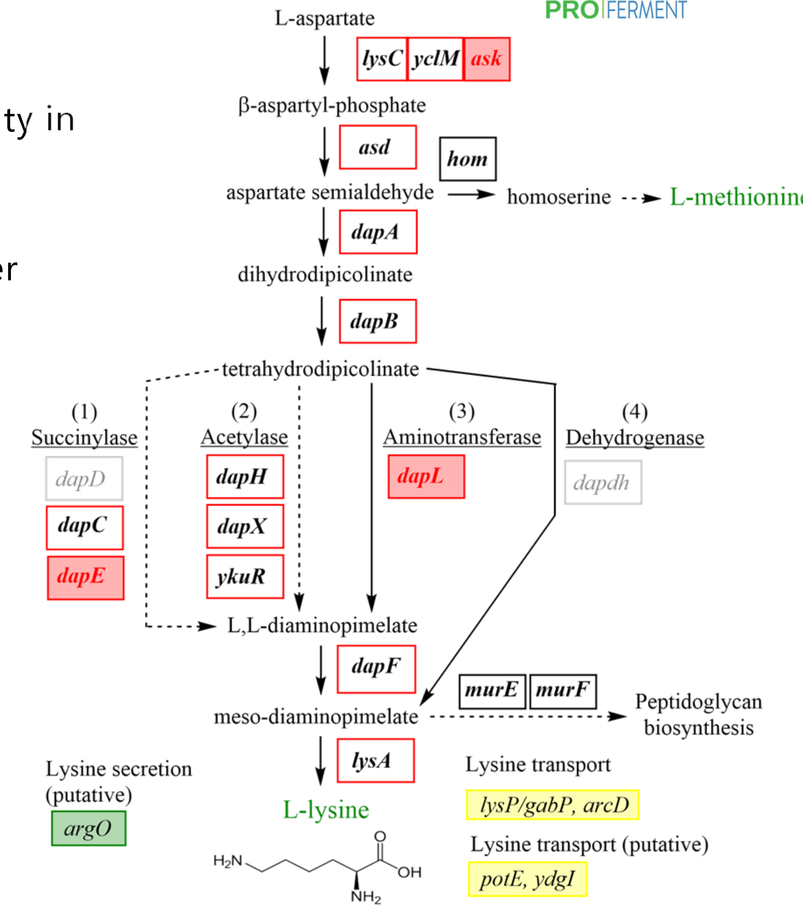
Lysine production by *Bacillus* and *Priestia* spp.

Fermentation with ***Bacillus*** and ***Priestia* spp.** can improve protein quality in plant-based foods through de-novo synthesis of L-lysine

L-lysine biosynthesis is limited by the expression of lysC**,** which is under **riboswitch control**

A **riboswitch** is a regulatory mRNA element that can regulate gene expression upon binding with specific metabolites

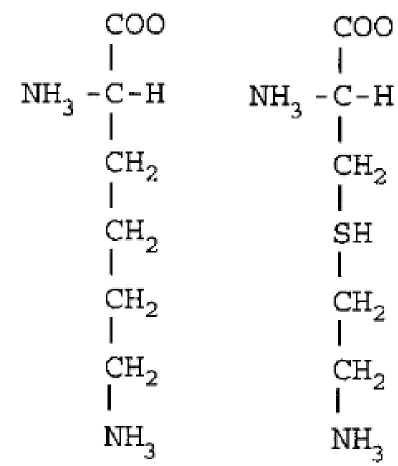




Canoy et al., npj Science of Food, accepted

How to force a cell to produce something it does not need without GMO'ing it?

Hypothesis: Increased **L-lysine** production can be achieved through chemical mutagenesis with lysine analogue **aminoethylcysteine** (**AEC**)

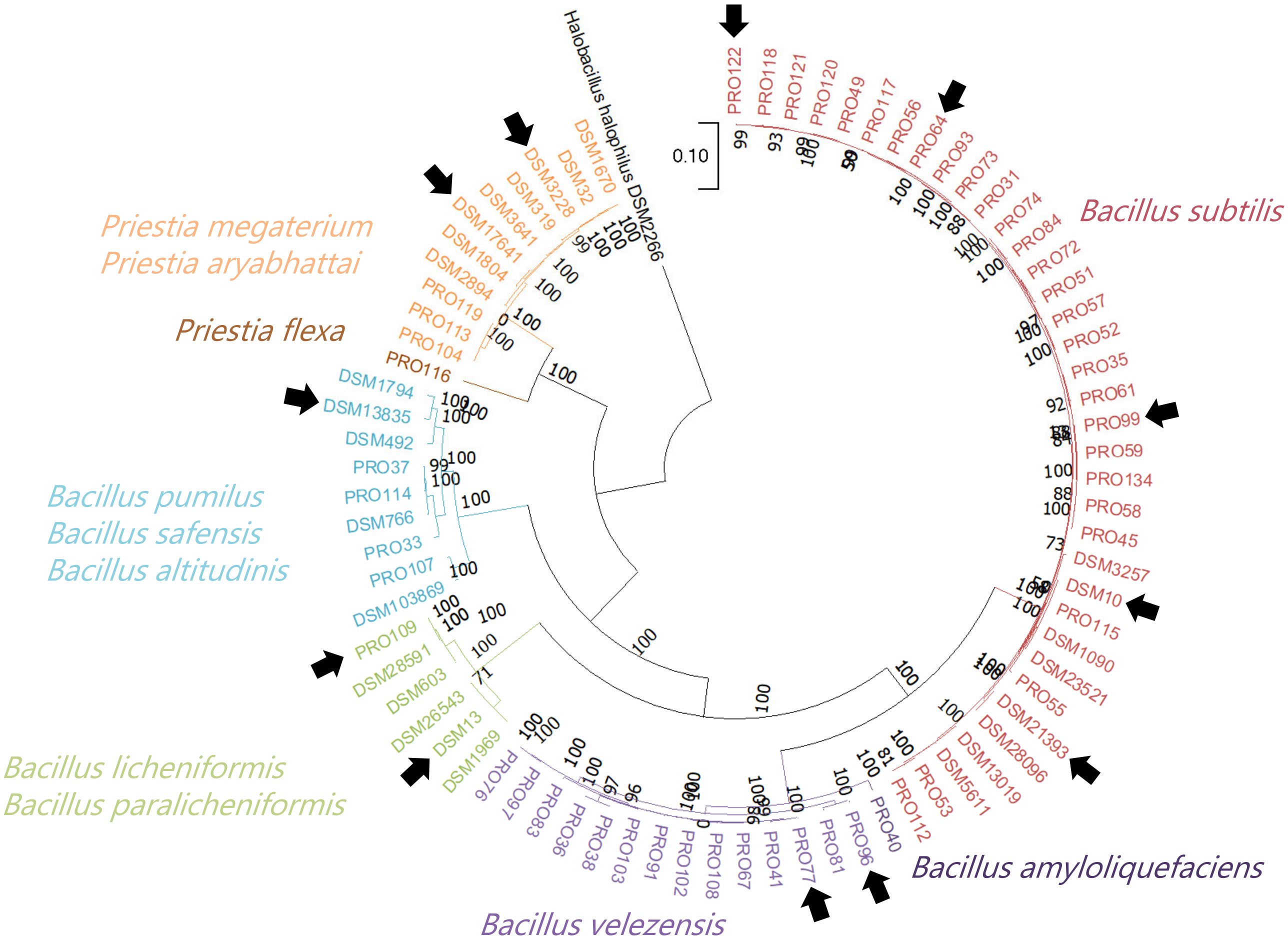


Lysine AEC

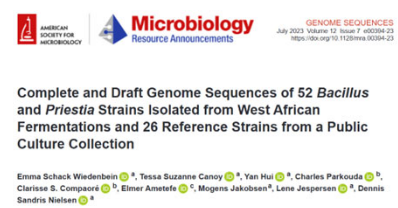
AEC resistant mutants of *Bacillus* spp. have been previously associated with mutations in the **lysC riboswitch** element

Canoy et al., npj Science of Food, accepted

Bacillus/Priestia culture collection and selection for AEC mutagenesis



Selected for AEC mutagenesis (11)


Microbiology Resource Announcements
GENOME SEQUENCES

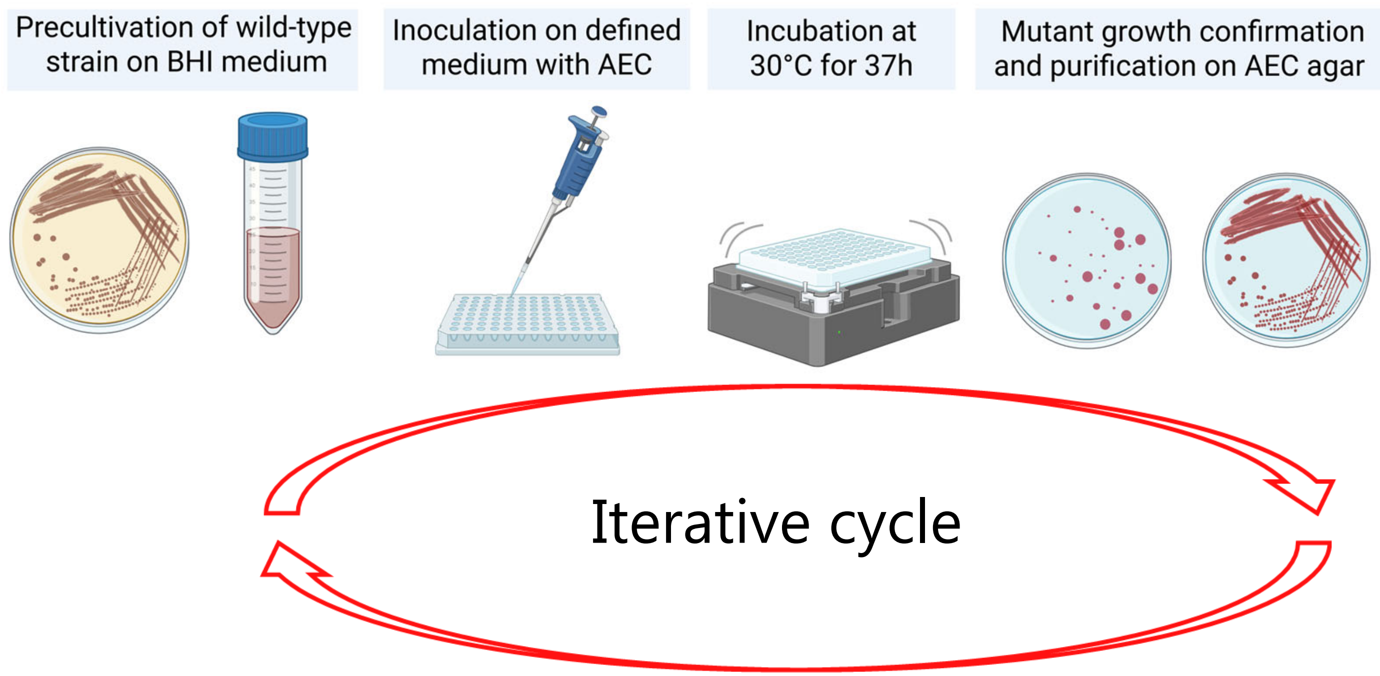
Complete and Draft Genome Sequences of 52 *Bacillus* and *Priestia* Strains Isolated from West African Fermentations and 26 Reference Strains from a Public Culture Collection

Emma Schack Wiedenbein[a], Tessa Suzanne Canoy[a], Yan Hui[a], Charles Parkouda[b], Clarisse S. Compaoré[b], Elmer Ametefe[c], Mogens Jakobsen[a], Lene Jespersen[a], Dennis Sandris Nielsen[a]

Canoy et al., npj Science of Food, accepted

How to generate lysine overproducing mutants?

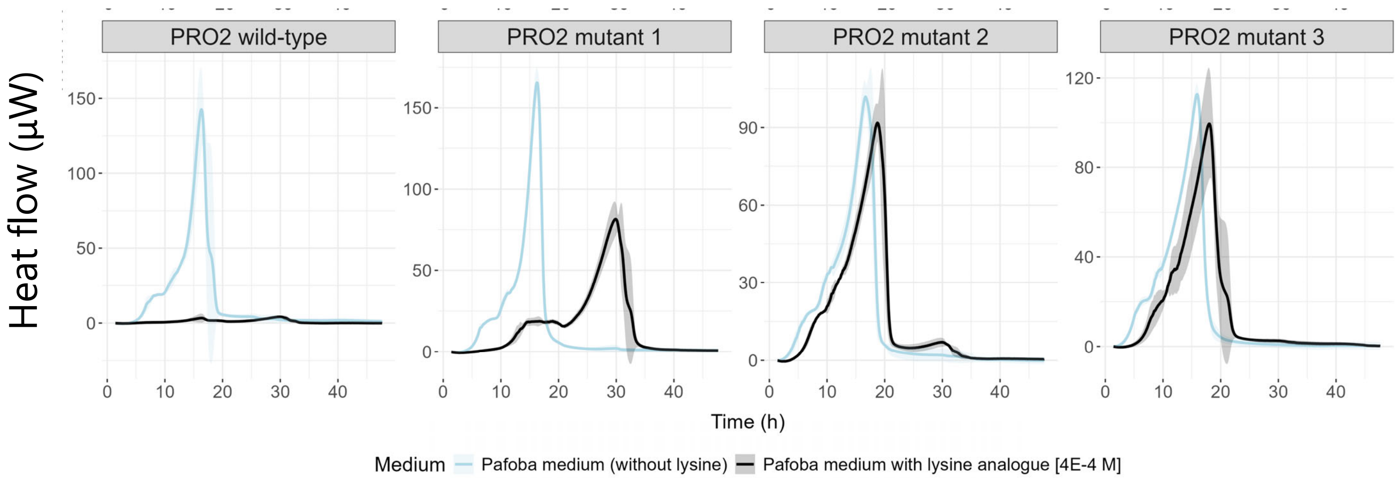
Mutant generation



Canoy et al., npj Science of Food, accepted

AEC-tolerance of *B. subtilis* wild-type and mutant strains

Growth of wild-type and mutants on substrate without (light blue) or with AEC (the lysine analogue)



Mutant strains show increased resistance to grow on AEC medium compared to the wild-types

Metabolic activity determined via calorimetry (calScreener)

Canoy et al., npj Science of Food, accepted

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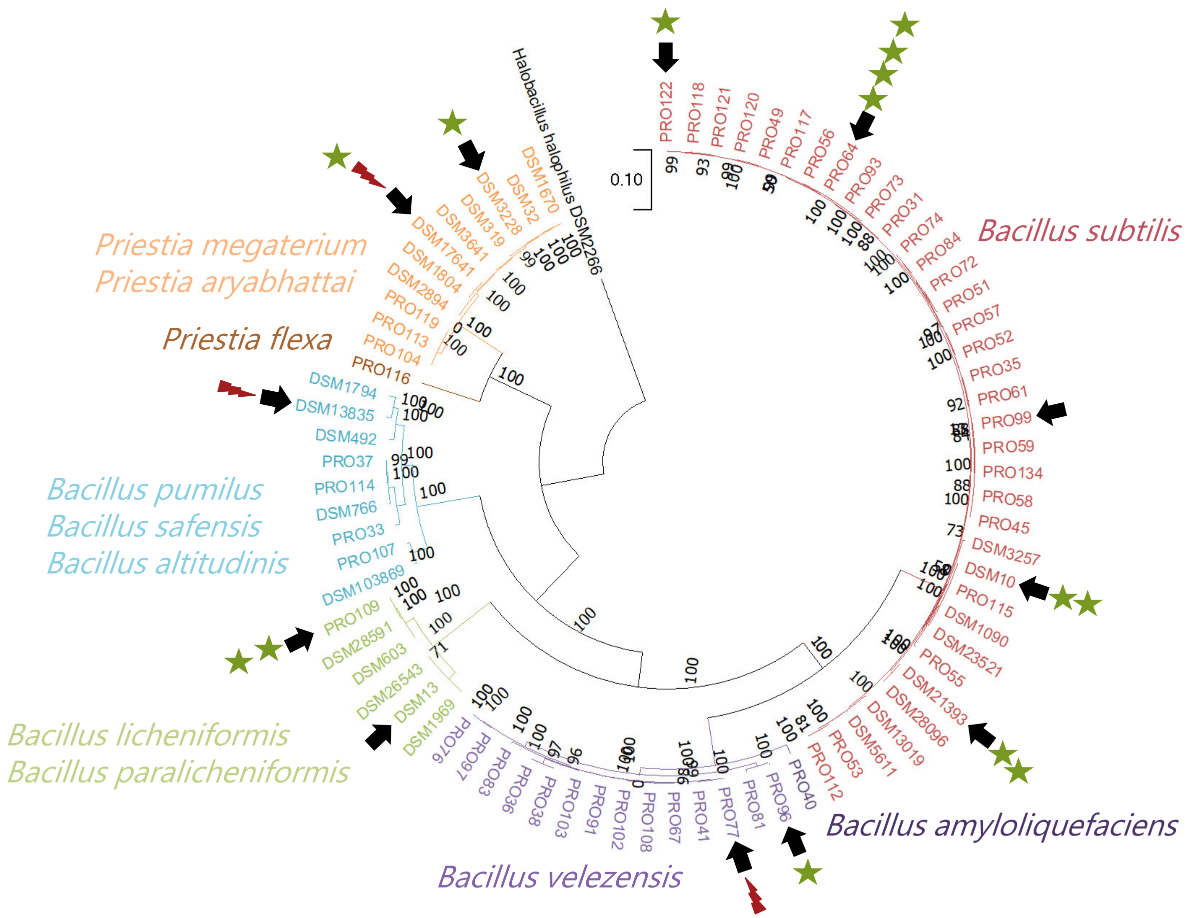
Bacillus/Priestia AEC-resistant mutants



From 11 wild-type strains we generated **21** AEC-resistant mutants

- Wild-types used for AEC mutagenesis (11)
- Mutants with lysC riboswitch mutation (3/21)
- Mutants with (putative) lysine transport mutation (14/21)

Canoy et al., npj Science of Food, accepted

AEC-induced mutations of lysC riboswitch

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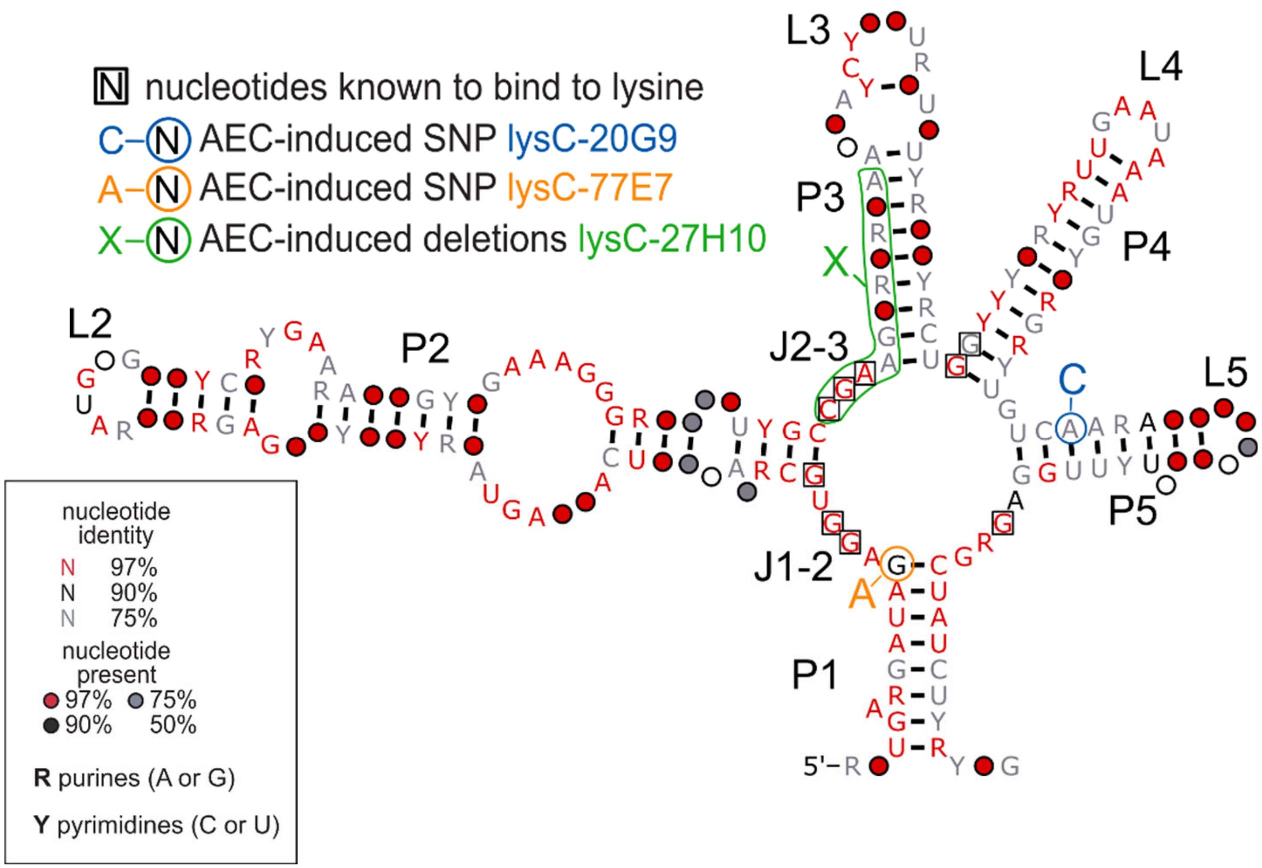
Consensus secondary structure from 30 *Bacillus* and *Priestia* lysine riboswitch sequences



Three mutants had changes in their lysC riboswitch structure, either single nucleotide polymorphism or deletions

Priestia megaterium 20G9
Bacillus velezensis 77E7
Bacillus pumilus 27H10

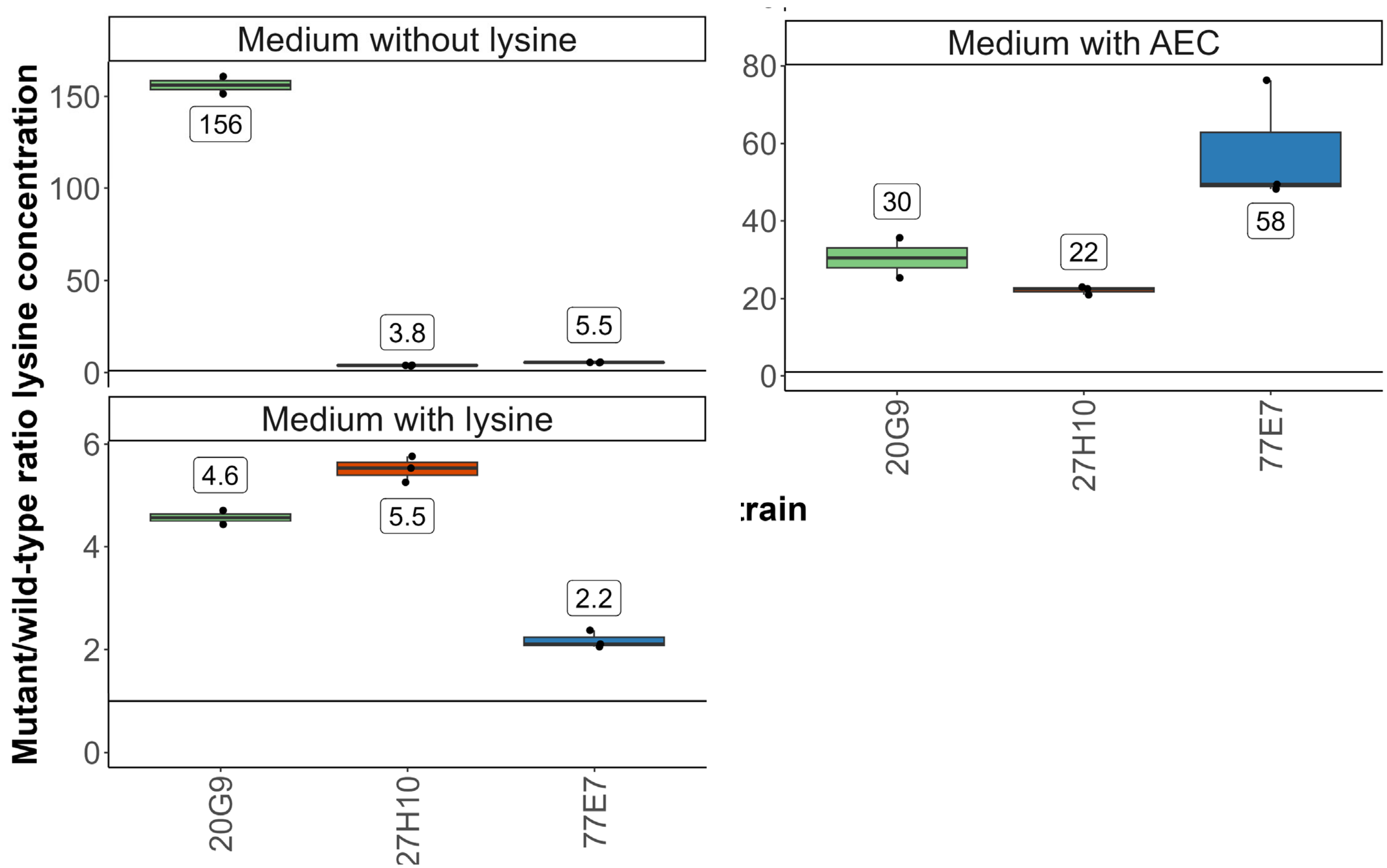
Mutants 20G9 and 27H10 have mutations close to the lysine binding nucleotides

Canoy et al., npj Science of Food, accepted

Mutants produce more lysine than wild-types regardless of medium

Compared to the wild-type, mutant *P. megaterium* 20G9 **produced 156x more lysine** in a medium without lysine

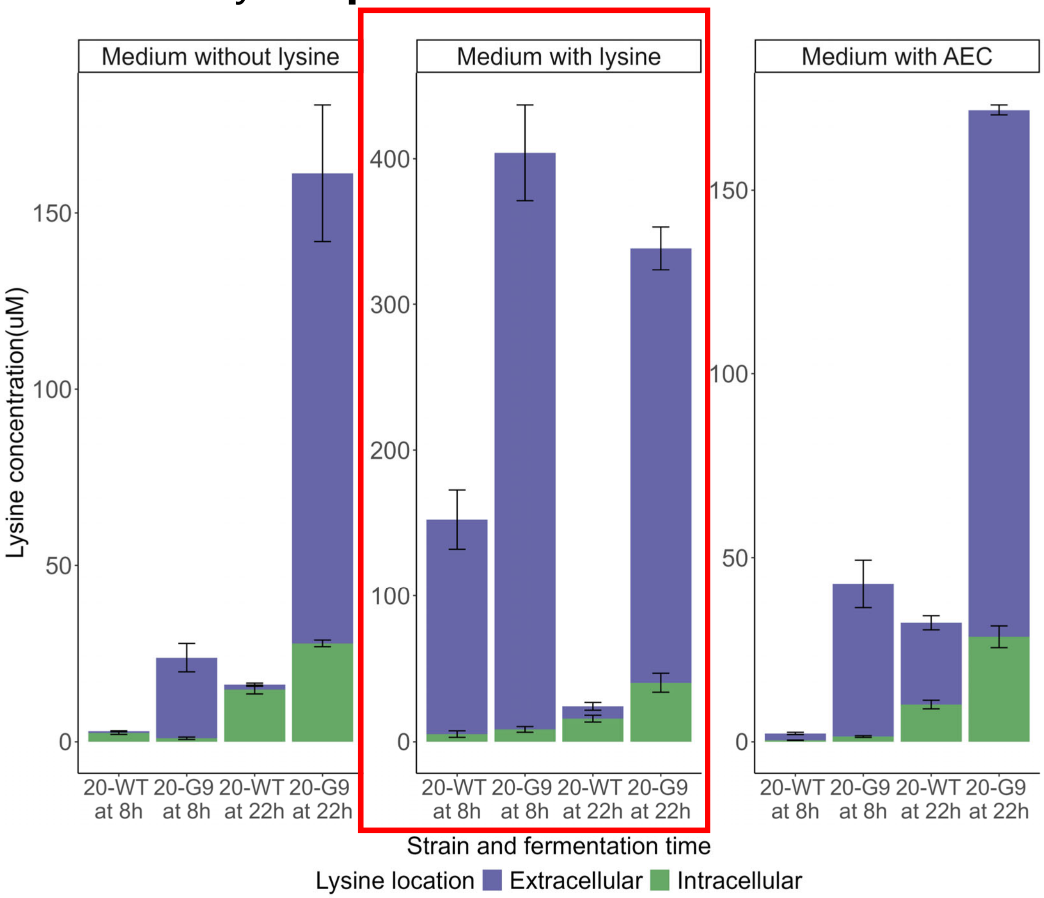
Compared to the wild-types, the AEC mutants **produced 2-6x more lysine** in a medium with lysine



Canoy et al., npj Science of Food, accepted

Liquid fermentation of 20-G9 and 20-WT

Lysine **production** at 8h and 22h



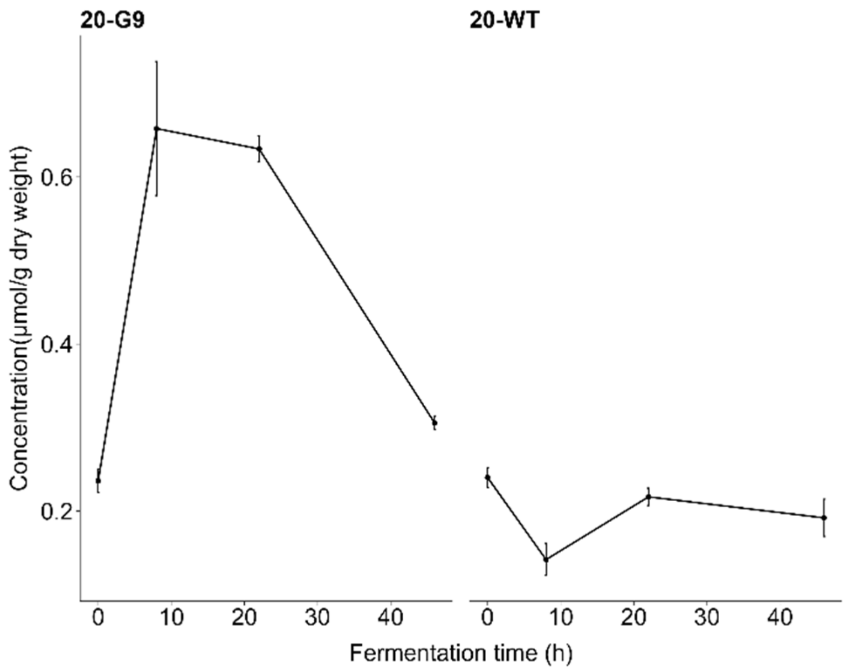
Riboswitch mutant 20-G9 produces **more intracellular (22h) and extracellular lysine (8h, 22h)** than its wild-type 20-WT

A large part of the lysine produced by riboswitch mutant 20-G9 is located **extracellularly**

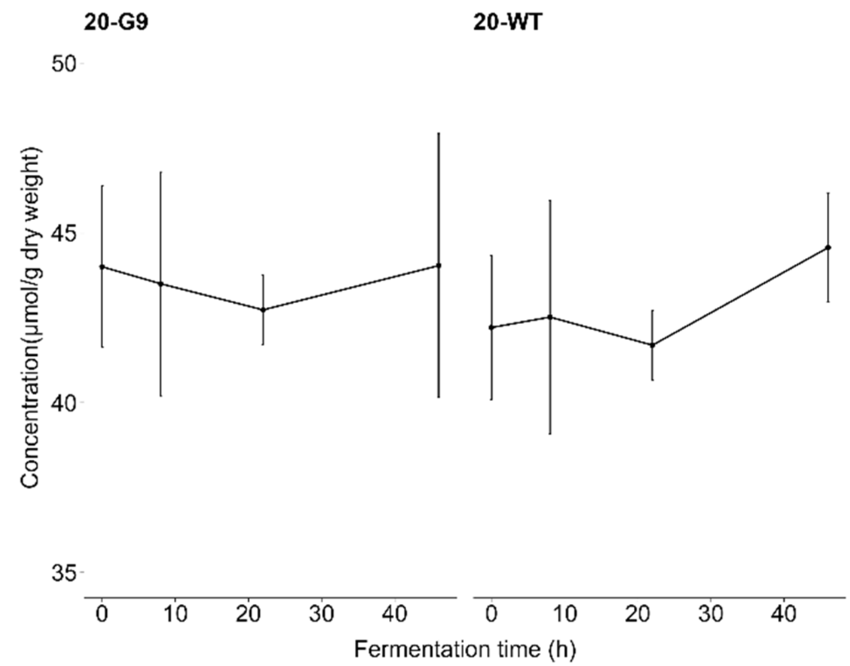
Canoy et al., npj Science of Food, accepted

Free and total lysine content after oat fermentation

Free lysine content



Total lysine content



Oat fermentation with mutant **20-G9** resulted in a higher **free lysine** content than with wild-type 20-WT. However, in the solid matrix no significant increase.

Significant lysine increase in broth/liquid. For solid fermentations more biomass needed

Canoy et al., npj Science of Food, accepted

How to get more biomass?

- Unpublished

Plant-based food materials do not contain vitamin B12

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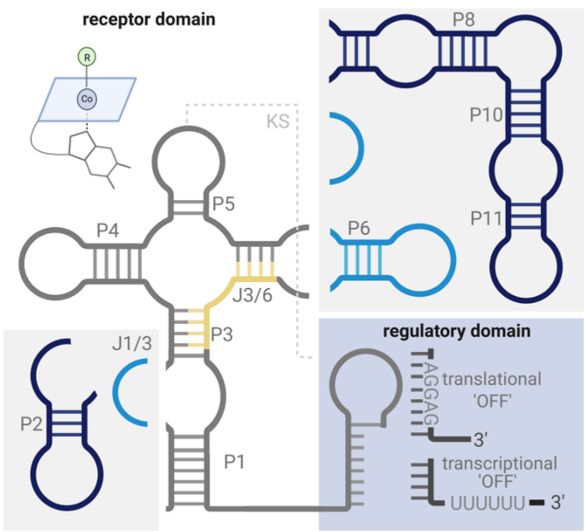
INDEPENDENT RESEARCH FUND DENMARK

- But bacteria can produce it for us, if we can tame them
  - *Propionibacterium freudenreichii* and *Priestia megaterium* are promising food grade candidates
- B12-synthesis is (similar to lysine) under riboswitch control
  - Complex four-way riboswitch junction with variable peripheral extensions – specific to the cobalamin derivative
- **Challenge:** Production seizes early: Biosynthesis stops at a threshold, likely due to riboswitch-mediated repression (unpublished data)
- We are now deciphering how to circumvent the riboswitch control



We are kind of almost there: B12-containing tempeh

- Tempeh is an Indonesian *Rhizopus* (mould) fermented food
- Traditionally soya-based, but can also be made from e.g. yellow peas
- *Unpublished results deleted*
- (and you will daily at the same time easily live up to the dietary guidelines ("kostråd") telling you to eat 100 gram legumes)

Stapput, Iversen et al., in prep.

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INDEPENDENT RESEARCH FUND DENMARK

Acknowledgements

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- The Novo Nordisk Foundation
- Independent Research Foundation Denmark
- Green Development and Growth Program (GUDP)

Thank you for your attention

I am happy to answer questions

- Now and/or via dn@food.ku.dk