U.PORTO

# Faba bean fermentation with lactic acid bacteria and *Bacillus subtilis*: sensory and nutritional impact

# *Fermentação de fava com bactérias ácido-lácticas e Bacillus subtilis: impacto sensorial e nutricional*

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**Abstract**

Faba bean (*Vicia faba L.*) is a promising sustainable protein source, but its use is limited by antinutritional factors and undesirable sensory properties. Microbial fermentation may address these limitations, with lactic acid bacteria (LAB) and *Bacillus subtilis* offering distinct metabolic capabilities. This study evaluated whether co-culture fermentation of LAB with *B. subtilis* Natto improves the nutritional and taste-relevant composition of faba bean beyond single-strain fermentation. Faba bean powder was fermented for 48 h with *Limosilactobacillus fermentum* UFLA CH 80, *Lactiplantibacillus plantarum* UFLA CH 93, and *B. subtilis* Natto, individually and in co-culture. Phytic acid, trypsin inhibitor activity, free amino acids, γ-glutamyl peptides, viable counts, and pH were measured. *B. subtilis* Natto produced the highest total free amino acid concentration (779.09 nmol/mL), including a 53-fold increase in lysine, and significantly reduced phytic acid and trypsin inhibitor activity. *L. fermentum* showed the highest numerical concentrations of six of the twelve γ-glutamyl peptides quantified. These complementary traits were not enhanced in co-culture: no combination outperformed the best-performing single strain, and partner-specific growth inhibition coincided with attenuation of several metabolic responses. These findings show that metabolic complementarity does not necessarily translate into co-culture synergy and highlight strain compatibility as a key determinant of fermentation performance.

**Keywords:** *Vicia faba;* Fermentation; Coculture Techniques; Lactic Acid Bacteria; *Bacillus subtilis*.

**Resumo**

A fava (*Vicia faba L.*) é uma fonte proteica sustentável promissora, mas a sua utilização é limitada por fatores antinutricionais e características sensoriais indesejáveis. A fermentação microbiana pode contribuir para ultrapassar estas limitações, sendo que as bactérias do ácido láctico (BAL) e *Bacillus subtilis* apresentam capacidades metabólicas distintas. Este estudo avaliou se a co-cultura de BAL com *B. subtilis* Natto melhora a composição nutricional e os compostos relevantes para o sabor da fava em comparação com estirpes únicas. O pó de fava foi fermentado durante 48 h com *Limosilactobacillus fermentum* UFLA CH80, *Lactiplantibacillus plantarum* UFLA CH93 e *B. subtilis* Natto, individualmente e em co-cultura. Foram determinados ácido fítico, atividade inibidora de tripsina, aminoácidos livres, péptidos γ-glutamil, células viáveis e pH. *B. subtilis* Natto produziu a maior concentração total de aminoácidos livres (779,09 nmol/mL), incluindo um aumento de 53 vezes na lisina, e reduziu significativamente o ácido fítico e a atividade inibidora de tripsina. *L. fermentum* apresentou as maiores concentrações numéricas em seis dos doze péptidos γ-glutamil quantificados. Estas características complementares não foram potenciadas em co-cultura: nenhuma combinação superou a melhor estirpe individual, e a inibição do crescimento coincidiu com a atenuação de várias respostas metabólicas. Os resultados mostram que a complementaridade metabólica não se traduz necessariamente em sinergia e destacam a compatibilidade entre estirpes como determinante do desempenho da fermentação.

**Palavras-Chave:** *Vicia faba;* Fermentação; Técnicas de Co-cultura; Bactérias ácido-láticas; *Bacillus subtilis*.

**List of abbreviations, acronyms and initialisms**

AOCS — American Oil Chemists' Society

ANOVA — Analysis of variance

BAPNA — Nα-Benzoyl-DL-arginine 4-nitroanilide

BHI — Brain Heart Infusion

CFU — Colony-forming units

FAA — Free amino acids

FBP — Faba bean powder

G6PD — Glucose-6-phosphate dehydrogenase

GGT — γ-Glutamyltransferase

HSD — Honestly significant difference

LAB — Lactic acid bacteria

LC— Liquid Chromatography

MRS — de Man, Rogosa and Sharpe

SD — Standard deviation

TCA — Trichloroacetic acid

TIA — Trypsin inhibitor activity

TUI — Trypsin units inhibited

UHPLC — Ultra-high-performance liquid chromatography

UHPLC–MS — Ultra-high-performance liquid chromatography–mass spectrometry

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## 1. Introduction

Faba bean (*Vicia faba L.*) is a promising sustainable protein source due to its high protein content, adaptation to temperate climates, and contribution to biological $N_2$ fixation [1-3]. Its wider use in human nutrition is nevertheless constrained by antinutritional factors and undesirable flavour. Phytic acid reduces mineral bioavailability [4], trypsin inhibitors impair protein digestion[5], and the pyrimidine glycosides vicine and convicine can trigger hemolytic anemia (favism) in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency[6]. Conventional processing methods such as soaking and cooking can reduce these compounds, although their effectiveness varies with the antinutrient and processing conditions [7, 8]. Lipid-oxidation-derived aldehydes, particularly hexanal and heptanal, further contribute to the characteristic green and beany flavour of faba bean [9, 10].

Microbial fermentation offers a biological strategy to modify both nutritional composition and flavour. Lactic acid bacteria (LAB) rapidly acidify food matrices and can metabolize aldehydes associated with beany off-flavour [11, 12]. Their proteolytic system relies mainly on cell-envelope proteinases, peptide transport and intracellular peptidases [13], whereas *Bacillus subtilis* possesses a broad repertoire of secreted extracellular proteases [14]. *B. subtilis* proteases can degrade proteinaceous antinutrients, including trypsin inhibitors [15], and extensive amino acid release has been reported during *Bacillus* fermentation of faba bean protein [16]. Fermentation can also modify taste-relevant metabolites: free amino acids such as glutamate contribute to umami perception, while γ-glutamyl peptides are associated with kokumi-related mouthfulness and taste

continuity[17, 18]. Microbial formation of γ-glutamyl peptides through γ-glutamyltransferase activity has also been demonstrated in LAB [19].

These distinct metabolic capacities provide a rationale for combining LAB and *B. subtilis*. However, microbial interactions in mixed cultures can modify both growth and metabolic activity, making co-culture performance difficult to predict from mono-culture behaviour [20, 21]. We therefore hypothesized that combining LAB with *B. subtilis* Natto could integrate complementary functions and improve the nutritional and taste-relevant composition of faba bean beyond single-strain fermentation. Faba bean powder was fermented with *Limosilactobacillus fermentum* UFLA CH80, *Lactiplantibacillus plantarum* UFLA CH93 and *B. subtilis* Natto, individually and in two-strain combinations, to compare their effects on microbial growth, pH, phytic acid, trypsin inhibitor activity, free amino acids and γ-glutamyl peptides.

## 2. Materials and Methods

### 2.1 Raw material and substrate preparation

Faba bean powder (FBP; Biogan, Denmark), containing 27.2% protein, 55.3% carbohydrates, 3.0% fat and 7.0% dietary fibre, was gamma-irradiated and milled by the supplier. Fermentation suspensions were prepared at 10% (w/v) in distilled water.

### 2.2 Microbial Strains and Culture Conditions

Three strains were used in this study: *Lactiplantibacillus plantarum* UFLA CH93 and *Limosilactobacillus fermentum* UFLA CH80, both originally isolated from Brazilian cocoa fermentations, and *Bacillus subtilis* Natto, obtained from

commercial natto spores. Stock cultures of the LAB were maintained and activated on de Man, Rogosa and Sharpe (MRS) agar at 30 °C for 48 h, whereas *B. subtilis* Natto was maintained on Brain Heart Infusion (BHI) agar at 30 °C for 24 h. Reagents were purchased from Sigma-Aldrich (Denmark) or Thermo Fisher Scientific (Denmark) unless otherwise stated.

### 2.3 Fermentation Design and Sampling

*B. subtilis* Natto was propagated in BHI broth at 30 °C, 200 rpm for 24 h, and LAB in MRS broth at 30 °C for 24 h. Cells were harvested, washed and resuspended in 0.9% NaCl. Mono-cultures were inoculated at $10^5$ CFU/mL, while each strain in co-cultures was inoculated at $10^5$ CFU/mL. Fermentations were conducted at 30 °C and 120 rpm with non-inoculated control. Samples were collected at 0 and 48 h for viable counts and pH; samples for biochemical analyses were stored at −60 °C and freeze-dried. Experiments were performed in biological triplicate.

### 2.4 Viable Cell Counts and pH Measurement

Viable cell counts (CFU) were determined by plating serial dilutions onto MRS agar for LAB and BHI agar for *B. subtilis* Natto. In co-cultures containing LAB and *B. subtilis* Natto, colonies were presumptively assigned according to growth on the respective culture medium together with characteristic colony morphology. In the *L. plantarum* + *L. fermentum* co-culture, the two LAB strains were differentiated based on their distinct colony morphotypes.

The pH of fermented samples was measured immediately after sampling using a FiveEasy Plus FP20 pH meter (Mettler Toledo, Glostrup, Denmark).

### 2.5 Phytic Acid Determination

Phytic acid was determined using the K-PHYT Phytic Acid/Total Phosphorus Assay Kit (Megazyme/Neogen, Ireland). Freeze-dried samples (0.5 g) were extracted with 0.66 M HCl for 3 h and subsequently neutralized with 0.75 M NaOH and enzymatically dephosphorylated with phytase and alkaline phosphatase to determine total phosphorus with a phytase-free reaction used to determine free phosphorus. Released phosphate was quantified at 655 nm, and phytic acid was calculated from the difference between total and free phosphorus and expressed as g/100 g.

### 2.6 Trypsin Inhibitor Activity Assay

Trypsin inhibitor activity (TIA) was determined according to AOCS Method Ba 12a-2020, with modifications based on Liu et al.[23]. Briefly, 1.0 mL of diluted extract was mixed with 2.5 mL BAPNA (0.4 mg/mL) and 1.0 mL trypsin (20 µg/mL), incubated at 37 °C for 10 min, and stopped with 0.5 mL of 30% acetic acid. Tubes were centrifuged, and absorbance was measured at 410 nm against a sample blank. TIA was expressed as trypsin units inhibited (TUI) per mg of sample.

### 2.7 Free Amino Acid Analysis

Free amino acids were extracted following Hildebrand et al.[24]. Briefly, 100 mg of freeze-dried sample was mixed with 5 mL of trichloroacetic acid (6.25% w/v) and incubated at 4 °C for 3 h, then centrifuged at 5,000 × g for 30 min. The supernatant was diluted 1:1 (v/v) with Milli-Q water, neutralized with 1 M NaOH,

spiked with 6-aminocaproic acid (50 µM) as an internal standard, and filtered through a 0.2 µm filter (Phenomenex, Denmark).

Quantification was performed using an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, CA, USA) equipped with an AdvancedBio AAA column (Agilent) and fluorescence detection, according to Schmidt et al. (2020). Results were expressed as nmol/mL.

### 2.8 γ-Glutamyl Peptides Analysis

γ-Glutamyl peptides were extracted from freeze-dried samples using an in-house protocol adapted from Li et al. [22]. Samples were homogenized with 0.5 mm glass beads using a FastPrep-24 5G instrument (MP Biomedicals, USA) at 6.5 m/s for 30 s, repeated three times. Proteins were precipitated with trichloroacetic acid, and the supernatant was acidified with 0.1% (v/v) trifluoroacetic acid and filtered through a 0.22 µm syringe filter. Peptides were analyzed by LC–MS using a Dionex Ultimate 3000 system coupled to a Q Exactive Orbitrap mass spectrometer and a BioZen Peptide XB-C18 column (1.7 µm, 150 × 2.1 mm; Phenomenex). Results were expressed as µmol/kg.

### 2.9 Statistical analysis

Treatments were performed in biological triplicate and analysed using IBM SPSS Statistics, with significance set at $p < 0.05$. Differences among treatments were assessed by one-way ANOVA followed by Tukey's HSD after Levene's test. Independent-, paired- and one-sample t-tests were used for two-group comparisons, 0–48 h paired measurements and comparison of pH treatments with

the single 48 h control, respectively. Data with fewer than three analytically valid replicates were reported descriptively or excluded from pairwise comparisons.

## 3. Results

### 3.1 Microbial growth and pH

In mono-culture fermentations, *B. subtilis* Natto and *L. fermentum* showed significant increases in viable counts from 7.20 to 8.30 and from 7.45 to 9.59 log CFU/mL, respectively, over 48 h. In contrast, *L. plantarum* showed a significant decrease from 9.97 to 9.16 log CFU/mL, despite having a substantially higher initial viable count than the other two strains (Figure 1A). In co-culture fermentations, *B. subtilis* Natto was not detected (< 2 log CFU/mL) in two of three replicates when combined with *L. fermentum*, whereas the remaining replicate reached 8.57 log CFU/mL and was not included in inferential testing (Figure 1B). In co-culture with *L. plantarum*, the viable count of *B. subtilis* Natto was numerically lower than in mono-culture (7.00 vs. 8.30 log CFU/mL), although the difference was not statistically significant. *L. plantarum* viable counts were significantly lower in co-culture with *B. subtilis* Natto (7.52 log CFU/mL) than in mono-culture (9.16 log CFU/mL) and in co-culture with *L. fermentum* (8.77 log CFU/mL). In contrast, *L. fermentum* viable counts did not differ significantly among mono- and co-culture conditions.

All three mono-cultures showed significantly different pH values from the 48 h unfermented control (6.38). *B. subtilis* resulted in a slightly higher final pH (6.92), whereas *L. fermentum* (4.53) and *L. plantarum* (4.16) resulted in substantially lower pH values, with *L. plantarum* showing the greatest difference from the unfermented control (Figure 2A). Among co-culture fermentations, pH at 48 h differed significantly from the unfermented control in two of the three

combinations. *L. fermentum* + *L. plantarum* resulted in the lowest final pH (4.22), followed by *B. subtilis* + *L. fermentum* (4.84). In contrast, *B. subtilis* + *L. plantarum* (6.28) did not differ significantly from the unfermented control (Figure 2B).

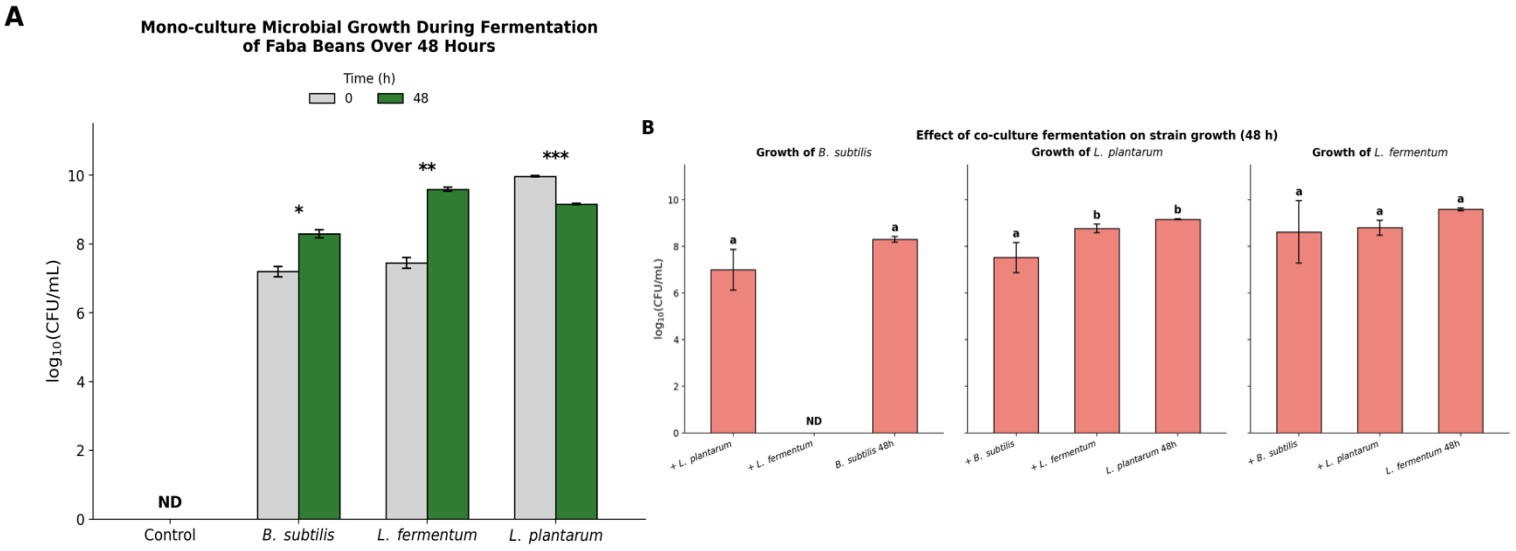


**Figure 1**. Viable counts (log CFU/mL). (A) Mono-culture growth from 0 h to 48 h. Values are means ± SD from triplicate experiments. Asterisks denote significant differences between 0 h and 48 h, determined by paired-samples t-test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001). (B) Effect of co-culture fermentation on strain growth at 48 h. Different lowercase letters above bars indicate statistically significant differences among conditions for the same strain, determined by one-way ANOVA followed by Tukey's HSD post hoc test (P ≤ 0.05). ND = not detectable (< 2 log CFU/mL) and was excluded from statistical testing.

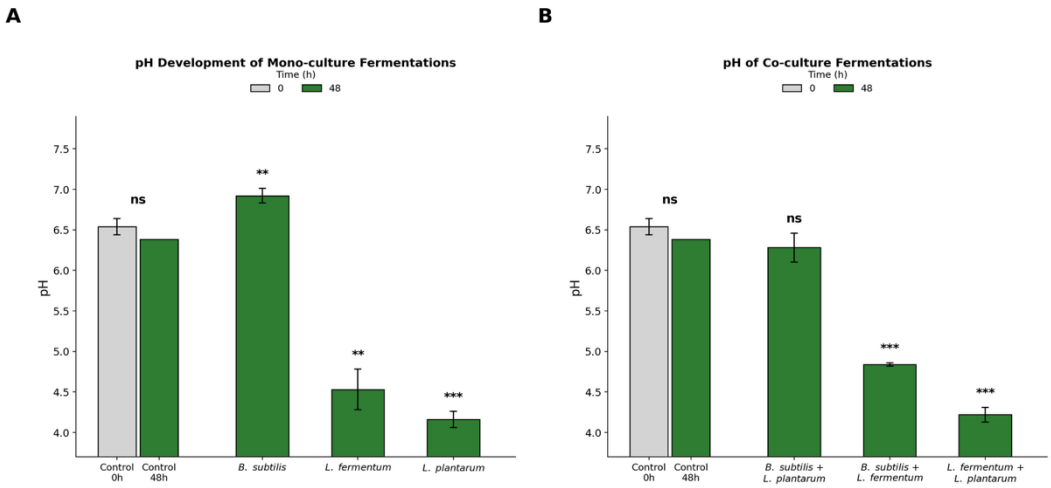


**Figure 2.** pH of the faba bean media during mono-culture and co-culture fermentation. (A) pH of mono-cultures of *Bacillus subtilis* Natto (BS), *Limosilactobacillus fermentum* UFLA CH80 (Lf) *and Lactiplantibacillus*

*plantarum* UFLA CH93 (Lp) after 48 h of fermentation. (B) pH of co-culture fermentations (BS + Lf, BS + Lp, and Lf + Lp) after 48 h. Data are presented as mean ± SD (n = 3), except for the 48 h control, a single measurement (n = 1) used as the fixed reference value for statistical comparisons. Significant differences from the 48 h control were determined by one-sample t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant).

### 3.2 Free amino acids

Free amino acid (FAA) concentrations before and after 48 h of fermentation are presented in Appendix 1 (Table 1). In the unfermented substrate, arginine was the predominant FAA (52.53 nmol/mL). Arginine decreased markedly with *B. subtilis* and *B. subtilis* + *L. plantarum*, while remaining unchanged with *L. plantarum*. Lysine showed the strongest response, increasing approximately 53-fold with *B. subtilis*, from 3.11 to 165.34 nmol/mL, while *B. subtilis* + *L. plantarum* showed an intermediate concentration. *B. subtilis* also produced the highest numerical concentrations of several other FAAs, although pairwise comparisons for some compounds were limited by insufficient valid replicates. Overall, *B. subtilis* produced the highest total FAA concentration (779.09 nmol/mL) and essential FAA concentration (560.54 nmol/mL).

### 3.3 γ-Glutamyl peptides

Concentrations of twelve γ-glutamyl peptides before and after 48 h of fermentation are presented in Appendix 2 (Table 2). Before fermentation, γ-Glu-Cys, γ-Glu-Met, and γ-Glu-His were the most abundant peptides (18.36, 7.39, and 2.67 µmol/kg, respectively), while the remaining peptides were ≤1.50 µmol/kg. *L. fermentum* showed the highest numerical concentrations of six peptides, notably γ-Glu-Leu (15.52 µmol/kg; ~11-fold above the unfermented substrate) and γ-Glu-Met (21.37 µmol/kg). γ-Glu-Gln was highest with *B. subtilis* (4.49 µmol/kg), while *B. subtilis* + *L. fermentum* also exceeded the unfermented substrate (2.24

vs. 1.50 µmol/kg). γ-Glu-Gly increased mainly in LAB-containing fermentations, reaching 6.08 µmol/kg with *L. plantarum* and 3.35 µmol/kg with *L. fermentum* + *L. plantarum*. γ-Glu-Cys was significantly higher in the unfermented substrate than in all fermented samples, whereas γ-Glu-Val and γ-Glu-His showed no significant pairwise differences.

### 3.4 Trypsin inhibitor activity

Trypsin inhibitor activity (TIA) differed significantly among treatments ($p < .001$), with all fermented groups showing significantly lower activity than the unfermented substrate (6.59 TUI/mg) (Figure 3). The lowest TIA was observed following fermentation with *B. subtilis* + *L. plantarum* (4.11 TUI/mg), although this value did not differ significantly from *B. subtilis* mono-culture (4.54 TUI/mg) or *B. subtilis* + L. fermentum (4.81 TUI/mg).

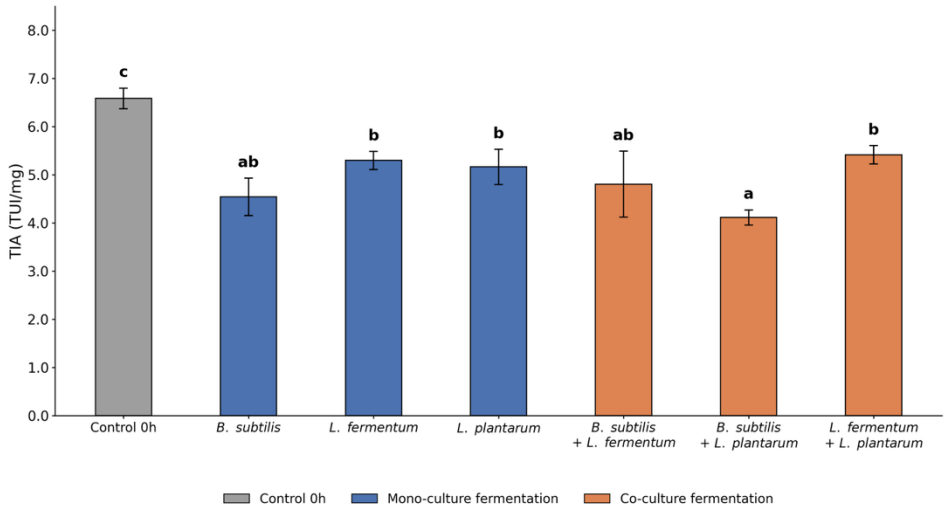


**Figure 3**. Trypsin inhibitor activity (TIA, TUI/mg) in faba bean media before fermentation (0 h) and after 48 h of fermentation with *Bacillus subtilis* Natto, *Limosilactobacillus fermentum* UFLA CH 80, *and Lactiplantibacillus plantarum* UFLA CH 93. Values are means ± SD from triplicate experiments. Different lowercase letters above bars indicate statistically significant differences among samples, as determined by one-way ANOVA followed by Tukey's HSD post hoc test ($p \leq 0.05$).

### 3.5 Phytic acid

Phytic acid content differed significantly among treatments ($p < .001$) (Figure 4). Compared with the unfermented substrate (2.47 g/100 g), significantly lower concentrations were observed following fermentation with *B. subtilis* (1.37 g/100 g) and *B. subtilis* + *L. plantarum* (1.47 g/100 g). In contrast, *L. fermentum* (2.31 g/100 g), *L. plantarum* (2.65 g/100 g), *B. subtilis* + *L. fermentum* (2.00 g/100 g), and *L. fermentum* + *L. plantarum* (2.70 g/100 g) did not differ significantly from the unfermented substrate.

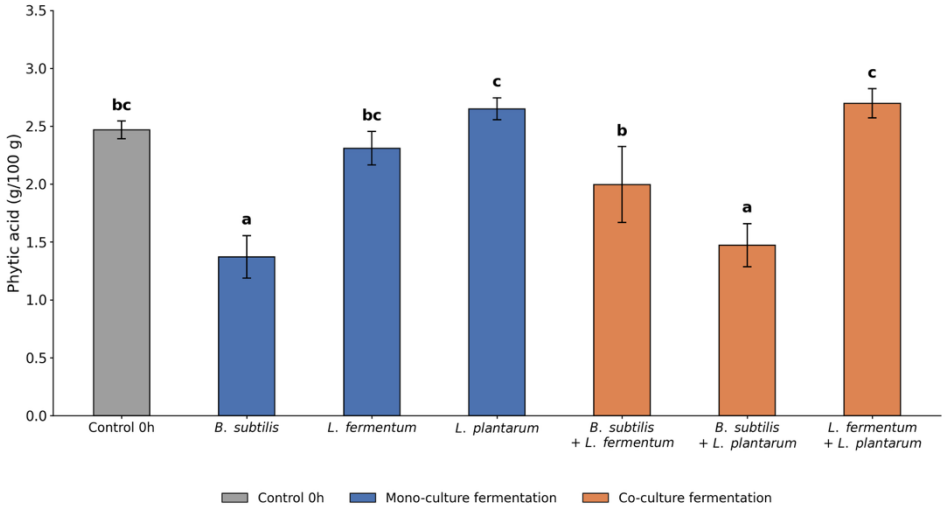


**Figure 4**. Phytic acid content (g/100 g) in faba bean media before fermentation (0 h) and after 48 h of fermentation with *Bacillus subtilis* Natto, *Limosilactobacillus fermentum* UFLA CH 80, and *Lactiplantibacillus plantarum* UFLA CH 93. Values are means ± SD from triplicate experiments. Different lowercase letters above bars indicate statistically significant differences among samples, as determined by one-way ANOVA followed by Tukey's HSD post hoc test ($p \leq 0.05$).

## 4. Discussion

This study investigated whether combining LAB with *B. subtilis* Natto in co-culture fermentation could improve the nutritional and taste-relevant properties of faba bean compared with single-strain fermentation. Under the conditions tested, none of the co-cultures consistently outperformed the best-performing mono-culture. Instead, performance depended strongly on the strain combination, indicating that metabolic complementarity alone was insufficient to ensure synergy. This

should not be interpreted as evidence that LAB–*Bacillus* co-culture is inherently unsuitable for faba bean fermentation, since consortium performance depends on strain compatibility and environmental conditions [25, 26]. The present findings therefore apply to the specific strains and simultaneous inoculation strategy evaluated here.

Microbial growth patterns helped explain several of the compositional outcomes. In mono-culture, *B. subtilis* and *L. fermentum* increased significantly in viable counts, whereas *L. plantarum* decreased despite its higher initial inoculum. The *B. subtilis* + *L. fermentum* interaction was particularly unstable: *B. subtilis* was not detected in two of three replicates, although high counts occurred in the remaining replicate. This co-culture also reached a markedly acidic final pH (4.84), consistent with reports that lactic acid production by LAB can inhibit *B. subtilis* in co-culture [26]. Because organic acids were not quantified and pH was not independently controlled, acidification cannot be separated from other competitive mechanisms. In contrast, *B. subtilis* remained at viable counts comparable to mono-culture when combined with *L. plantarum*, while *L. plantarum* counts were significantly reduced. These partner-specific interactions were reflected in the metabolite profiles: where *B. subtilis* remained well established, several of its characteristic effects were retained, whereas its unstable growth with *L. fermentum* coincided with attenuation of those responses. Species assignment in co-culture relied on selective media and colony morphology rather than strain-specific molecular confirmation, which should be considered when interpreting small differences in viable counts.

*B. subtilis* produced the strongest FAA response, increasing total FAAs from 109.01 to 779.09 nmol/mL and markedly enriching several essential amino acids. This pattern is consistent with extensive protein degradation and amino acid release reported during natto and faba bean fermentation [27, 16]. The approximately 53-fold increase in lysine further illustrates the magnitude of this FAA response. However, FAA concentrations reflect not only amino acid release but also their subsequent microbial metabolism. Arginine, for example, was strongly depleted by *B. subtilis* but remained unchanged with *L. plantarum*, consistent with strain-dependent arginine utilization reported by Larsen et al.[16]. Importantly, the *Bacillus*-associated FAA response was attenuated in co-culture: *B. subtilis* + *L. plantarum* produced an intermediate FAA pool, whereas the pronounced lysine response was lost with *L. fermentum*, paralleling the inconsistent establishment of *B. subtilis* in that combination. Trypsin inhibitor activity decreased significantly in every fermented treatment. The lowest numerical value occurred with *B. subtilis* + *L. plantarum*, but it did not differ significantly from *B. subtilis* alone or *B. subtilis* + *L. fermentum*; LAB mono-cultures also reduced TIA, consistent with Coda et al.[28]. Proteolysis is a plausible mechanism, although faba bean trypsin inhibitor has been characterized as Bowman–Birk-type proteins [29], whereas Sharmila et al.[15] demonstrated degradation of a Kunitz-type inhibitor by a *B. subtilis* bacillopeptidase. Thus, the latter supports the capacity of *B. subtilis* proteases to inactivate trypsin inhibitors rather than identifying the specific mechanism operating here. Interestingly, *B. subtilis* + *L. plantarum* retained low TIA despite producing substantially fewer FAAs than *B. subtilis* alone, suggesting that TIA reduction did not simply parallel overall amino acid release.

Phytic acid showed a more strain-specific response. Significant reductions occurred only with *B. subtilis* and *B. subtilis* + *L. plantarum*, consistent with the phytase-producing capacity of natto strains [30]. Since *L. plantarum* alone did not reduce phytic acid, while *B. subtilis* maintained comparable viable counts in the co-culture, the effect is most consistently associated with *B. subtilis*. Coda et al.[28] similarly reported no significant phytate reduction following *L. plantarum* fermentation of faba bean flour. Phytate reduction was retained when *B. subtilis* remained well established with *L. plantarum* but lost when its establishment was inconsistent with *L. fermentum*, mirroring the lysine response. The effective treatments also remained closer to neutral pH, compatible with the activity range reported for several *Bacillus* phytases [31], although phytase activity was not measured directly.

The γ-glutamyl peptide profile differed markedly from the FAA pattern. Despite producing a much smaller FAA pool than *B. subtilis*, *L. fermentum* showed the highest numerical concentrations of six of the twelve γ-glutamyl peptides quantified, including γ-Glu-Leu and γ-Glu-Met, whereas *B. subtilis* was most prominent for γ-Glu-Gln. Thus, peptide accumulation did not simply follow the extent of amino acid release. γ-Glutamyltransferases (GGTs) can generate these peptides by transferring γ-glutamyl groups to amino-acid or peptide acceptors [32], and LAB-mediated formation of γ-glutamyl dipeptides has been demonstrated experimentally [19]. Because GGT activity was not measured, the pathway remains a mechanistic interpretation rather than a directly demonstrated response. Nevertheless, the contrasting FAA and γ-glutamyl profiles reveal distinct

metabolic contributions from *B. subtilis* and *L. fermentum*, consistent with species-dependent differences reported during faba bean fermentation [16].

Several peptides elevated by *L. fermentum* are also relevant to taste. γ-Glu-Leu has been identified as a kokumi-active compound in edible beans, contributing to mouthfulness, complexity and taste continuity [33], while other peptides detected here have been associated with similar kokumi-related properties in fermented foods [34]. Kokumi perception has also been linked to activation of the calcium-sensing receptor [18]. The present chemical data therefore indicate potential taste relevance, but no sensory evaluation or receptor-based assay was performed, so perceptible sensory enhancement cannot be inferred. In addition, γ-Glu-Cys decreased across all fermented treatments, illustrating that endpoint concentrations reflect the balance between peptide formation and turnover. Time-resolved profiling and direct GGT measurements would be needed to resolve these dynamics.

The comparison between *B. subtilis* and *L. fermentum* is particularly relevant to consortium design. In mono-culture, the former strongly promoted amino acid release and phytate reduction, whereas the latter favoured accumulation of several γ-glutamyl peptides. These traits were therefore potentially complementary, but simultaneous co-culture failed to integrate them. *B. subtilis* + *L. plantarum* retained selected *Bacillus*-associated effects, particularly low TIA and reduced phytic acid, without outperforming the mono-culture and with a smaller FAA pool. In contrast, *B. subtilis* + *L. fermentum* showed inconsistent *Bacillus* establishment, loss of its pronounced lysine response and absence of significant phytate reduction, while several γ-glutamyl peptides were also lower than with *L. fermentum* alone. Thus, one co-culture preserved selected functions

without generating additional benefit, whereas the other attenuated characteristic functions of both partners.

## 5. Conclusion

This study demonstrated distinct metabolic contributions of B. subtilis Natto and LAB during faba bean fermentation. *B. subtilis* Natto produced the strongest FAA response and reduced phytic acid, whereas *L. fermentum* generated the highest concentrations of several γ-glutamyl peptides with potential taste relevance; all fermentations reduced trypsin inhibitor activity. However, no co-culture consistently outperformed the best-performing mono-culture, indicating that metabolic complementarity did not translate into synergy under simultaneous inoculation. Interpretation is limited by the small strain panel and predominantly endpoint measurements. Future work should investigate broader strain combinations, alternative inoculum ratios and sequential inoculation.

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# Appendices

## Appendix 1. Free amino acid concentrations in faba bean powder before and after 48 h of fermentation

**Table 1.** Concentration of free amino acids (FAAs, nmol/mL) in faba bean media before fermentation (0 h) and after 48 h of fermentation with *Bacillus subtilis* Natto, *Limosilactobacillus fermentum* UFLA CH80, and *Lactiplantibacillus plantarum* UFLA CH93.
*Mean values (± SD) from triplicate experiments are shown, except where noted (N = number of valid replicates). nd = not detected. — = excluded from analysis. Different lowercase superscript letters within the same row indicate statistically significant differences among samples, as determined by one-way ANOVA followed by Tukey's HSD post hoc test (P ≤ 0.05). Rows without letters had at least one group with fewer than two valid replicates, precluding post hoc comparison.*

| FAAs | Concentration, nmol/mL | | | | | | |
|---|---|---|---|---|---|---|---|
| | Before fermentation, 0h | After 48 h fermentation | | | | | |
| | | *B. subtilis* | *B. subtilis* + *L. fermentum* | *B. subtilis* + *L. plantarum* | *L. fermentum* | *L. fermentum* + *L. plantarum* | *L. plantarum* |
| Asp | 12.43±1.13 | 1.40±1.31 | nd | nd | nd | nd | 9.88±1.37 |
| Glu | 27.82±0.41 | 42.45±11.29 | 12.12±2.22 | 27.93±2.06 | 0.43 | 39.36±5.46 | 42.52±3.46 |
| Ser | nd | 0.90 | nd | nd | 0.42±0.12 | nd | nd |
| His | nd | 44.85±11.47 | 0.78±0.63 | 16.39±4.29 | nd | nd | 0.001 |
| Gly | 5.43±0.42$^{a}$ | 16.61±6.03$^{b}$ | 18.34±2.47$^{b}$ | 5.70±1.14$^{a}$ | 14.78±0.94$^{b}$ | 25.86±0.84$^{c}$ | 28.29±3.09$^{c}$ |
| Thr | nd | nd | nd | nd | nd | nd | nd |
| Arg | 52.53±0.38 | 3.23±1.25 | nd | 2.14±1.08 | nd | nd | 54.46±6.44 |
| Ala | 3.84±0.26$^{a}$ | 90.12±16.30$^{c}$ | 61.32±7.29$^{b}$ | 57.09±4.85$^{b}$ | 103.44±6.81$^{c}$ | 38.58±10.71$^{b}$ | 13.30±1.01$^{a}$ |
| Tyr | nd | 64.29±14.22 | 1.80±0.76 | 30.99±7.45 | nd | nd | nd |
| Val | nd | 101.68±9.51 | 3.68±1.27 | 50.56±10.20 | nd | nd | nd |
| Met | — | — | — | — | — | — | — |
| Trp | 3.13±0.27 | 98.70±2.09 | 8.50±1.28 | 49.65±10.84 | nd | 4.20±0.88 | 5.15±0.19 |
| Phe | nd | 30.05±7.07 | nd | 8.10±3.36 | nd | nd | nd |
| Ile | nd | nd | nd | nd | nd | nd | nd |
| Leu | nd | 119.91±25.40 | 10.53±1.17 | 48.83±13.44 | nd | 6.39±1.60 | 8.83±0.22 |
| Lys | 3.11±0.20$^{a}$ | 165.34±32.35$^{c}$ | 22.24±2.32$^{a}$ | 92.69±14.02$^{b}$ | 7.83±1.19$^{a}$ | 11.24±1.67$^{a}$ | 10.46±0.05$^{a}$ |
| Gln | nd | nd | nd | nd | nd | nd | nd |
| **Essential FAA** | **6.97±0.43** | **560.54±106.71$^{c}$** | **45.73±6.67$^{a}$** | **263.52±53.36$^{b}$** | **7.83±1.19$^{a}$** | **21.83±4.04$^{a}$** | **24.44±0.38$^{a}$** |
| **Total FAA** | **109.01±1.48** | **779.09±157.73$^{c}$** | **139.31±19.41$^{a}$** | **387.37±64.42$^{b}$** | **126.19±8.98$^{a}$** | **125.91±11.52$^{a}$** | **172.89±15.44$^{a}$** |

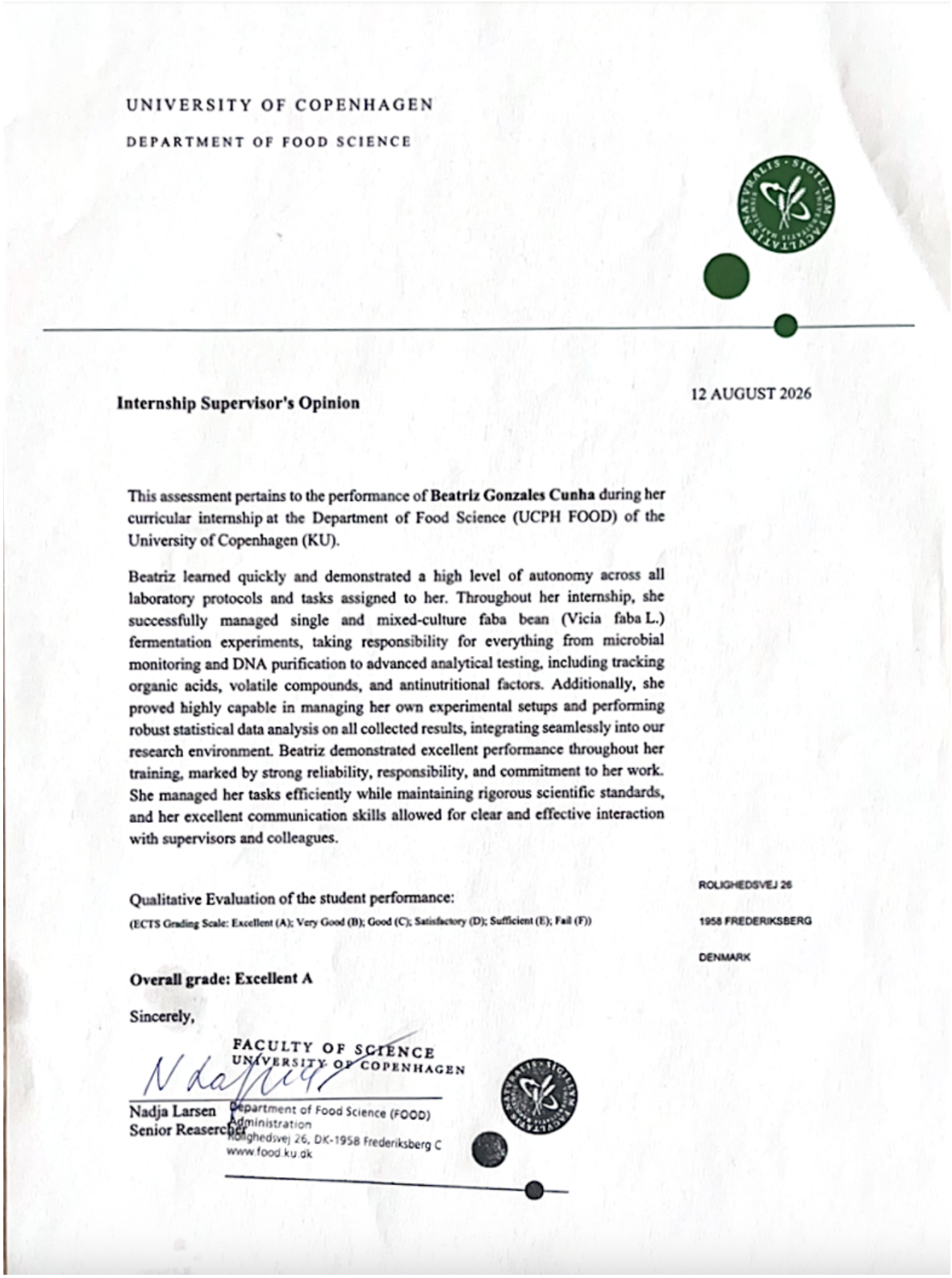
## Appendix 2. γ-Glutamyl peptide concentrations in faba bean powder before and after 48 h of fermentation

**Table 2. Concentration of γ-glutamyl peptides (µmol/kg) in faba bean media before fermentation (0 h) and after 48 h of fermentation with *Bacillus subtilis* Natto, *Limosilactobacillus fermentum* UFLA CH 80, and *Lactiplantibacillus plantarum* UFLA CH 93.**

*Mean values (± SD) from triplicate experiments are shown. Different lowercase superscript letters within the same row indicate statistically significant differences among samples, as determined by one-way ANOVA followed by Tukey's HSD post hoc test ($P \leq 0.05$). Rows without letters showed no significant difference among groups (γ-Glu-His, γ-Glu-Val).*

| γ-Glutamyl peptides | Concentration, µmol/kg | | | | | | |
|---|---|---|---|---|---|---|---|
| | Before fermentation, 0h | After 48 h fermentation | | | | | |
| | | *B. subtilis* | *B. subtilis* + *L. fermentum* | *B. subtilis* + *L. plantarum* | *L. fermentum* | *L. fermentum* + *L. plantarum* | *L. plantarum* |
| γ-Glu-His | 2.67±0.05 | 11.85±1.50 | 7.55±11.09 | 3.16±1.23 | 0.85±0.10 | 0.90±0.10 | 0.79±0.02 |
| γ-Glu-Gly | 0.253±0.006$^{a}$ | 0.19±0.01$^{a}$ | 0.257±0.058$^{a}$ | 0.24±0.044$^{a}$ | 1.213±0.397$^{b}$ | 3.35±0.442$^{c}$ | 6.083±1.181$^{c}$ |
| γ-Glu-Gln | 1.50±0.42$^{a}$ | 4.49±1.11$^{b}$ | 2.24±1.45$^{b}$ | 1.55±0.24$^{a}$ | 0.41±0.11$^{a}$ | 0.47±0.06$^{a}$ | 0.40±0.08$^{a}$ |
| γ-Glu-Glu | 0.020±0.001$^{ab}$ | -0.007±0.006$^{a}$ | 0.065±0.035$^{ab}$ | 0.003±0.006$^{a}$ | 0.303±0.247$^{b}$ | 0.147±0.012$^{ab}$ | 0.190±0.072$^{ab}$ |
| γ-Glu-Ala | 1.313±0.025$^{ab}$ | 0.257±0.036$^{ab}$ | 0.603±0.368$^{ab}$ | 0.537±0.246$^{ab}$ | 0.617±0.129$^{ab}$ | 0.227±0.116$^{a}$ | 1.493±0.935$^{b}$ |
| γ-Glu-Cys | 18.357±0.509$^{d}$ | 2.79±0.805$^{b}$ | 1.647±0.552$^{ab}$ | 0.825±0.049$^{a}$ | 4.343±0.621$^{c}$ | 1.973±0.257$^{ab}$ | 1.567±0.391$^{ab}$ |
| γ-Glu-Val | 0.310±0.020 | 1.490±0.304 | 1.287±1.605 | 0.337±0.127 | 0.733±0.059 | 0.507±0.006 | 0.510±0.010 |
| γ-Glu-Met | 7.39±0.41$^{a}$ | 4.34±0.30$^{a}$ | 9.61±4.75$^{a}$ | 4.47±2.16$^{a}$ | 21.37±5.00$^{b}$ | 5.22±0.24$^{a}$ | 5.07±0.34$^{a}$ |
| γ-Glu-Tyr | 0.67±0.26$^{a}$ | 1.00±0.26$^{ab}$ | 1.15±0.90$^{ab}$ | 0.31±0.07$^{a}$ | 1.74±0.21$^{b}$ | 1.32±0.05$^{ab}$ | 1.31±0.08$^{ab}$ |
| γ-Glu-Leu | 1.363±0.252$^{a}$ | 2.623±0.692$^{ab}$ | 9.22±1.69$^{c}$ | 0.960±0.157$^{a}$ | 15.52±1.43$^{d}$ | 4.51±0.20$^{b}$ | 4.40±0.21$^{b}$ |
| γ-Glu-Phe | 1.257±0.351$^{ab}$ | 2.543±0.775$^{ab}$ | 3.127±2.203$^{ab}$ | 0.627±0.182$^{a}$ | 3.65±0.244$^{b}$ | 2.023±0.096$^{ab}$ | 1.967±0.031$^{ab}$ |
| γ-Glu-Trp | 0.907±0.051$^{c}$ | 0.547±0.035$^{ab}$ | 0.663±0.102$^{b}$ | 0.447±0.006$^{a}$ | 1.537±0.110$^{d}$ | 1.407±0.051$^{d}$ | 1.397±0.051$^{d}$ |

## Opinion of the supervisor of the complementary work



UNIVERSITY OF COPENHAGEN

DEPARTMENT OF FOOD SCIENCE

**Internship Supervisor's Opinion**

12 AUGUST 2026

This assessment pertains to the performance of **Beatriz Gonzales Cunha** during her curricular internship at the Department of Food Science (UCPH FOOD) of the University of Copenhagen (KU).

Beatriz learned quickly and demonstrated a high level of autonomy across all laboratory protocols and tasks assigned to her. Throughout her internship, she successfully managed single and mixed-culture faba bean (Vicia faba L.) fermentation experiments, taking responsibility for everything from microbial monitoring and DNA purification to advanced analytical testing, including tracking organic acids, volatile compounds, and antinutritional factors. Additionally, she proved highly capable in managing her own experimental setups and performing robust statistical data analysis on all collected results, integrating seamlessly into our research environment. Beatriz demonstrated excellent performance throughout her training, marked by strong reliability, responsibility, and commitment to her work. She managed her tasks efficiently while maintaining rigorous scientific standards, and her excellent communication skills allowed for clear and effective interaction with supervisors and colleagues.

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DENMARK

Qualitative Evaluation of the student performance:

(ECTS Grading Scale: Excellent (A); Very Good (B); Good (C); Satisfactory (D); Sufficient (E); Fail (F))

**Overall grade: Excellent A**

Sincerely,

FACULTY OF SCIENCE
UNIVERSITY OF COPENHAGEN

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