PhD THESIS

Stability of phenolic-rich apple pomace during processing and evaluation of the *in vitro* prebiotic potential, antioxidant, and antiproliferative effects

PhD student **Diana Plămadă**

(SUMMARY OF THE DOCTORAL THESIS)

Scientific coordinator **Prof. univ. dr. Dan Cristian Vodnar**

SUMMARY

The global apple industry generates significant quantities of by-products, particularly apple pomace (AP), which remains largely underutilized despite its high content of bioactive compounds. Apple pomace, the solid residue left after juice extraction, is a rich source of dietary fiber and polyphenols, including flavonoids, phenolic acids, and dihydrochalcones. These compounds have been widely recognized for their potential health benefits, including antioxidant, prebiotic, and antiproliferative properties. However, despite its promising biofunctional attributes, AP is primarily used as animal feed or discarded, highlighting the need for innovative valorization strategies to enhance its applicability in human nutrition and functional food development.

Polyphenols, which constitute a significant fraction of AP's bioactive composition, play a crucial role in modulating gut microbiota, acting as prebiotic substrates that selectively promote beneficial bacterial growth. Several studies have demonstrated that phenolic-rich AP exhibits prebiotic effects, stimulating the proliferation of probiotic strains such as *Lactobacillus plantarum*, *Lactobacillus casei*, and *Saccharomyces boulardii*. These interactions suggest that AP may contribute to gut health by fostering microbial balance while exerting antimicrobial effects against enteric pathogens such as *Escherichia coli*. Moreover, the polyphenols in AP possess strong antioxidant activity, counteracting oxidative stress and reducing inflammation, which are key factors in various chronic diseases.

Beyond its prebiotic and antioxidant roles, recent research has highlighted AP's antiproliferative potential, particularly against colorectal cancer cells. *In vitro* assays have shown that specific polyphenols within AP can induce selective cytotoxicity, inhibiting cancer cell proliferation while having minimal impact on healthy fibroblasts. This effect is hypothesized to occur through multiple mechanisms, including oxidative stress modulation, apoptosis induction, and interference with cancer cell signaling pathways. These findings underscore the potential of AP as a functional ingredient that bridges gut health promotion with chemopreventive properties.

This study systematically evaluates the processing stability and biological activity of phenolic-rich AP, with a focus on its *in vitro* prebiotic potential, antioxidant properties, and antiproliferative effects. By characterizing the impact of processing methods such as freeze-drying on phenolic retention and bioactivity, this research contributes to optimizing AP utilization for functional food applications. The findings reinforce the importance of AP as a sustainable ingredient with multifaceted health benefits, supporting its integration into dietary interventions aimed at improving gut microbiota composition and reducing disease risk.

Therefore, the study highlights the importance of fully utilizing AP, aligning with the principles of a circular economy and sustainable food production. By optimizing its bioactive properties, AP can be transformed from an underutilized by-product into a valuable functional ingredient, reducing food waste while contributing to health-supporting dietary solutions. The methodologies and insights gained from this research can be extended to other fruit-derived by-products, further enhancing their nutritional value and applicability in the broader healthcare and nutrition sectors.

The main objectives of this thesis were:

The main focus of this Ph.D. thesis is the valorization of apple pomace (AP), an abundant agro-industrial by-product, into a high-value functional ingredient with prebiotic, antioxidant, and antiproliferative properties. By investigating its phenolic composition, biological activity, and potential applications, this research aims to contribute to the sustainable utilization of fruit-processing by-products within a circular economy framework. Several specific objectives have been established to systematically explore and validate the functional potential of AP. These objectives are:

O1. Characterization of phenolic composition and stability:

Analyze the phenolic profile of AP from different apple cultivars using advanced chromatographic techniques, assessing the impact of processing and preservation methods on phenolic stability and bioavailability.

O2. Evaluation of antioxidant and cytotoxic properties:

Investigate the antioxidant potential of AP extracts before and after *in vitro* digestion and fermentation, alongside assessing their selective cytotoxic effects on human colorectal cancer cells to explore their potential as functional food ingredients with health-promoting benefits.

O3. Assessment of prebiotic potential in *in vitro* models:

Examine the ability of freeze-dried AP to support the growth of selected probiotic microorganisms, including *Lactobacillus plantarum*, *Lactobacillus casei*, and *Saccharomyces boulardii*, by evaluating microbial viability and metabolic activity during fermentation.

O4. Optimization of AP valorization for functional applications:

Identify optimal processing strategies to preserve or enhance the prebiotic, antioxidant, and antiproliferative properties of AP, providing insights for its potential integration into functional food formulations and nutraceutical applications.

By achieving these objectives, this Ph.D. thesis aims to advance the understanding of AP's biofunctional properties and support its transition from a low-value by-product to a valuable dietary ingredient with significant health benefits. Additionally, this research aligns with broader efforts to reduce food waste and promote the sustainable use of agro-industrial residues.

Methodology

This study employed a combination of analytical, microbiological, and *in vitro* cell-based techniques to comprehensively evaluate the functional properties of apple pomace (AP). The research was structured into three primary experimental phases: (1) characterization of phenolic composition, stability, and processing effects, (2) evaluation of prebiotic potential and fermentation, and (3) assessment of antioxidant activity and selective cytotoxicity against tumoral cells.

Phenolic composition, stability, and processing impact

The phenolic profile of AP extracts was characterized using high-performance thin-layer chromatography (HPTLC) and liquid chromatography-mass spectrometry

(LC-MS/MS) to quantify key bioactive compounds, including flavonoids, phenolic acids, and dihydrochalcones.

To assess the impact of processing methods, AP extracts were subjected to thermal treatment (TT), high-pressure thermal treatment (HPTT), and pulsed electric field (PEF) treatment. TT involved heating at 80°C (T80) and 121°C (T121), while HPTT combined 600 MPa hydrostatic pressure with varying temperatures (50°C, 80°C, and 121°C). PEF treatment applied 10.9 kV, 30 Hz frequency, and 300 pulses to the extracts. Following these treatments, the stability of phloridzin, chlorogenic acid, and epicatechin was monitored to determine the effects on phenolic integrity and potential oxidative degradation.

Prebiotic potential and microbial fermentation

The prebiotic potential of AP was assessed through *in vitro* fermentation using three well-characterized probiotic strains: *Lactobacillus plantarum* ATCC 8014, *Lactobacillus casei* ATCC 393, and *Saccharomyces boulardii* MYA-796. Additionally, enteric strains of *Escherichia coli* (ATCC 25922 and ATCC 8739) were included to evaluate prebiotic selectivity.

To simulate digestion, AP was subjected to *in vitro* gastrointestinal digestion using the INFOGEST protocol, which mimics oral, gastric, and intestinal phases. After digestion, AP was incorporated into MRS and YPD media, replacing glucose at 0.5% and 1% w/w concentrations. Fermentation was conducted over 48 hours, with samples collected at 0, 4, 8, 24, and 48 hours to assess microbial growth and viability. The Prebiotic Index (PI) was calculated to compare AP effectiveness against fructooligosaccharides (FOS), a well-established prebiotic control.

Antioxidant and cytotoxic activity

To evaluate the antioxidant activity of AP extracts, three complementary assays were used: DPPH radical scavenging assay (which measures oxidative stress reduction), Trolox Equivalent Antioxidant Capacity (TEAC) assay, and Electron Paramagnetic Resonance (EPR) spectroscopy.

The cytotoxic potential of AP extracts against colorectal cancer cells was assessed using MTT and Trypan Blue exclusion assays on Caco-2 (human colorectal adenocarcinoma), and Hs27 (normal human fibroblast) cells. Cells were exposed to increasing concentrations (0–100 μg/mL) of AP extracts, and IC50 values were calculated to determine selective cytotoxic effects. Additionally, apoptosis assays were performed to confirm whether AP extracts induced cancer cell-specific toxicity.

Results

Phenolic composition and processing impact

The HPTLC and LC-MS/MS analysis identified a total of 19 phenolic compounds across three primary classes: flavonoids, phenolic acids, and dihydrochalcones. The phenolic content varied by apple cultivar, with Jonathan (JN) apple pomace exhibiting the highest procyanidin B1 content (0.69 mg/g DM), while Red Delicious (RD) pomace had the highest total phenolic content (TPC) at 13.81 mg GAE/g DM. In contrast, Granny

Smith (GS) pomace, due to its higher acidity, demonstrated enhanced antioxidant properties post-digestion.

Regarding processing impact, TT at 121°C (T121) and HPTT at 121°C (H121) led to 60–70% degradation of chlorogenic acid and epicatechin, confirming that higher temperatures accelerate phenolic degradation. Conversely, PEF treatment, although it resulted in a 50% reduction of phloridzin, preserved most other phenolics better than high-temperature treatments. Among all tested methods, low-temperature HPTT (H50) and PEF treatments were the most effective in maintaining phenolic stability.

Prebiotic potential and microbial fermentation

The prebiotic potential of AP was moderate, with selective support for probiotic growth, particularly *L. casei* and *S. boulardii*, which exhibited optimal fermentation patterns. The fermentation process resulted in a significant increase in short-chain fatty acids (SCFAs), particularly acetate and propionate, which are known to support gut health.

A key observation was that glucose and fructose levels decreased significantly during digestion, indicating that these sugars were readily utilized by beneficial gut bacteria. The Prebiotic Index (PI) scores showed that AP at 1% concentration with *Saccharomyces boulardii* exhibited the highest prebiotic activity (1.15 ± 0.20), surpassing other probiotic strains. However, AP was still less effective than FOS, suggesting that additional modifications or combinations with other prebiotics may enhance its functional benefits.

Antioxidant and cytotoxic properties

Post-digestion analysis revealed a 15% increase in DPPH inhibition, indicating that phenolic compounds became more bioavailable after digestion. The TEAC assay showed a 30–40% increase in antioxidant activity following mild thermal processing (T80, H50), while high-temperature HPTT (H121) significantly reduced antioxidant potential, likely due to extensive phenolic degradation.

Regarding cytotoxicity, AP extracts displayed selective inhibitory effects on colorectal cancer cells. The IC50 for Caco-2 cells was 41.36 μg/mL, whereas the IC50 for normal fibroblast Hs27 cells was 74.67 μg/mL, yielding a selectivity coefficient of 1.81. This indicates that AP extracts are preferentially toxic to cancer cells while sparing normal cells, suggesting a potential role in nutraceutical applications. Further analysis confirmed that AP extracts induced apoptosis in Caco-2 cells, reinforcing their potential anti-cancer properties.

The general conclusions of this thesis were:

1. The findings highlight that apple pomace (AP), a major by-product of the apple processing industry, possesses significant biofunctional properties, including prebiotic, antioxidant, and antiproliferative effects. This research underscores its potential as a functional ingredient, contributing to waste reduction while promoting human health.
2. Phenolic compounds in AP remain bioactive through various processing and digestive conditions, demonstrating stability that supports their application in

functional foods. The study confirmed that processing methods influence the phenolic profile, with specific apple cultivars showing higher concentrations of bioactive compounds.

3. The antioxidant activity of AP was shown to increase following *in vitro* digestion, suggesting that digestive processes enhance the bioavailability of certain phenolic compounds. This reinforces the potential role of AP in counteracting oxidative stress, a major factor in chronic diseases.
4. The selective cytotoxicity of AP extracts against colorectal cancer cells indicates its potential role in cancer prevention strategies. The observed preferential toxicity toward malignant cells, while sparing normal cells, supports further investigation into AP-derived bioactives for nutraceutical applications.
5. The prebiotic potential of AP was demonstrated through its ability to selectively support the growth of beneficial microorganisms, such as *Lactobacillus plantarum*, *Lactobacillus casei*, and *Saccharomyces boulardii*. However, prebiotic index scores suggest that while AP promotes probiotic growth, its effect is moderate compared to established prebiotics like fructooligosaccharides.
6. The study revealed that different apple cultivars exhibit variations in phenolic content and sugar composition, which in turn influence their prebiotic efficacy. These findings emphasize the importance of cultivar selection when considering AP as a functional ingredient.
7. Sustainable utilization of AP aligns with circular economy principles by transforming an underutilized waste product into a value-added ingredient. Optimizing processing techniques can enhance its functional properties, ensuring better integration into food and health applications.

Future research should explore long-term dietary interventions to validate AP's health benefits in human models. Additionally, optimizing processing strategies to enhance its prebiotic effects and bioactive stability will be crucial for developing AP-based functional foods and nutraceuticals.

Originality and personal contributions:

This Ph.D. thesis introduces several original contributions to the field of sustainable food valorization, particularly in the functional exploitation of apple pomace (AP), an agro-industrial by-product with significant bioactive potential. The research provides novel insights into the physicochemical properties of AP, its prebiotic potential, antioxidant stability, and antiproliferative effects, contributing to a deeper understanding of its applications in functional foods and nutraceuticals.

A major contribution of this research lies in the detailed characterization of AP-derived phenolic compounds across different apple cultivars. By employing advanced analytical techniques, this study demonstrated the cultivar-dependent variations in phenolic composition, sugar content, and their respective impacts on biological activity.

The findings establish a foundation for optimizing AP-based ingredients based on their biofunctional potential.

This research was among the few that systematically assessed AP polyphenols' stability and bioactivity following *in vitro* digestion and microbial fermentation. The study revealed that digestive processes enhance the bioavailability of certain phenolic compounds, increasing their antioxidant activity, which holds significance for their physiological effects post-consumption. Additionally, the fermentation trials provided valuable insights into the prebiotic effects of AP, showing its ability to selectively promote the growth of beneficial gut microorganisms such as *Lactobacillus casei* and *Saccharomyces boulardii*, albeit with a lower prebiotic index compared to commercial prebiotics.

A pioneering aspect of this thesis is the investigation of AP’s antiproliferative effects against colorectal cancer cells, demonstrating its selective cytotoxicity while sparing normal fibroblasts. The mechanistic insights proposed, including oxidative stress induction and apoptosis modulation, highlight the potential of AP-derived phenolics as functional components in cancer prevention strategies.

By demonstrating the multifunctional properties of AP, this research reinforces its role as a sustainable ingredient in functional food formulations, aligning with circular economy principles. The methodologies developed for assessing AP’s bioactivity can be adapted to other fruit-derived by-products, extending their applicability in waste valorization strategies.

Future research directions arising from this work include refining processing techniques to enhance the bioavailability of AP polyphenols, optimizing AP incorporation into food matrices, and conducting *in vivo* studies to further validate its health benefits. The integration of AP into sustainable food systems represents a promising avenue for reducing food waste while contributing to public health and environmental sustainability.