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Non-antibiotic anti-microbial effect Of Nutritional Additives

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CHAPTER 1

Foreword

1. Foreword

The widespread use of in-feed antibiotics in livestock production systems to prevent disease and metabolic disorders has raised concerns about the cross-resistance of pathogens to antibiotics in humans. Therefore, a ban on the antibiotics use in the European Union started from 2006. Post weaning period is a major critical period in the life of piglets, creep-feeding and weaning increase susceptibility to gut disorders, infections and diarrhea etc. therefore, more and more effort has been devoted towards developing alternatives to antibiotics.

The gut microbiota is a complex population, the initial colonization of the piglet gastrointestinal (GI) tract are aerobes and facultative anaerobes. Due to the high number of aerobic bacteria, consuming oxygen and reducing the redox potential, 48h after birth 90%of the total bacteria are strict anaerobes, including mainly lactobacilli and streptococci. Some of the dominant bacterial genera found during in suckling are Clostridium, Bacteroides and Bifidobacterium. Diet changes make the alternation of microbiota composition after weaning. The fermentable substrates reduced and the strict anaerobes increased, another important change is a decrease in lactobacilli species at the same time as enterobacteria increase in abundance. All these alterations result in an increased susceptibility to pathogenic bacteria.

Two to three weeks after weaning, the gut microbiota develops and becomes stable and diverse. This resident microbiota confers many benefits to the intestinal physiology of the host. Some of these benefits include the metabolism of nutrients and organic substrates, and the contribution to the colonization resistance to resist invasion by exogenous microorganisms.

It is well known that bacterial numbers differ greatly between the small and the large intestine. With respect to cultivable bacteria, the lower gastrointestinal tract mainly gram-positive anaerobes: streptococci, lactobacilli, eubacteria, clostridia and peptostreptococci at levels of 10^4–10^8 CFU/mL. In contrast, the upper GI tract: stomach, duodenum, jejunum and ileum, mainly lactobacilli and streptococci. The most heavily colonized region, however, is the colon, with a total population of 10^{11}–10^{12}CFU/ml of contents. Even though the composition of the GI tract microbiota remains quite stable during life, it is now well established that changes in the diet can cause changes in microbial activity and composition. Hence, managing of the diet is a viable way towards the identification of alternatives to add to in-feed antibiotics.

1.1 Plants extracts

The Plant polyphenols is one of the most powerful groups of bioactive compounds with antimicrobial and antioxidant property, which represents a diverse group of compounds (Hammer et al. 1999; Heim et al. 2002). The commonly available plant polyphenols involve green tea polyphenol, grape polyphenol, apple polyphenol and olive oil polyphenols, etc. Although some polyphenols are considered to be non-nutritive, interest in these compounds has arisen because of their possible beneficial effects on health. It has already been reported that polyphenols from olive leaf exert superb antimicrobial activity (Paul et al. 1997), tea polyphenols improve gut microflora balance in calves (Ishihara et al. 2001) and inhibit the proliferation of Chlamydia (Yamazaki et al. 2003), apple polyphenol extracts prevent damage to human gastric epithelial cells (Graziani1et al. 2005), and polypheols from grape seeds could prevent oxidative damage to cellular DNA in vitro (Fan and lou, 2004). A considerable amount of evidence concerning the immunomodulatory function of plant polyphenols has also been found (Hughes, 2005). Furthermore, it is believed that the efficacy of the antimicrobial compound will not be compromised due to the development of pathogen resistance. The British Pharmacopoeia (1996 Edition) reports that microorganisms do not build resistance to benzyl alcohol, phenols, polyphenols, and similar products.

1.2 Organic acid

As Organic acid has been used for many years for improving animal performance and to prevent digestive problems. The addition of organic acid generally lowers the pH and increases GI acidity, promotes beneficial bacteria and decrease pathogens. A study was shown that feeding benzoic acid to weaning pigs improved piglets' performance and was associated with a greater ileal microbiota biodiversity. Some of the most used organic acidifiers include formic, acetic, propionic, butyric, lactic, sorbic, fumaric, malic, and citric acid, Ca-formate, Ca-lactate, Ca-propionate, K-diformate, Ca-butyrate, Mg-citrate and Na-lactate.

1.3 Phytase

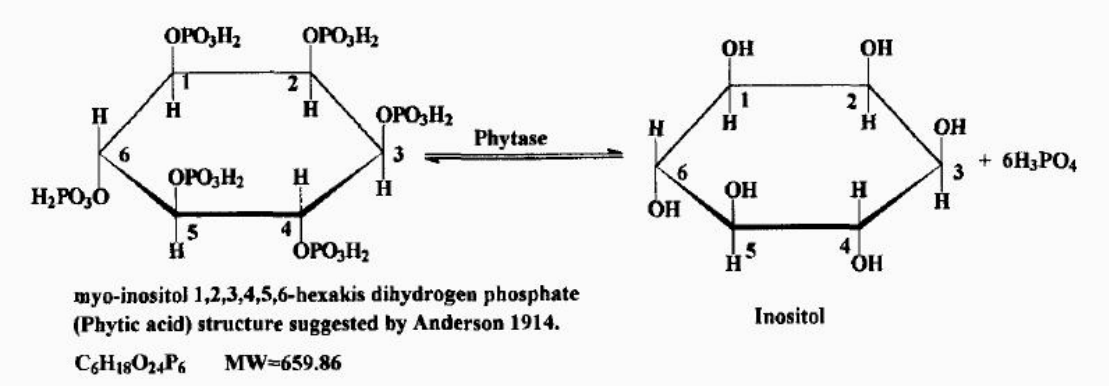

Figure 1 Hydrolysis of phytic acid to inositol and phosphoric acid by phytase.

Phytase is added in animal feeds to improve phosphorus nutrition and reduce phosphorus excretion of swine and poultry in areas of intensive animal agriculture. The commercial use research way of phytase has been explored for a long time. Phytase is produced by plants, bacteria, and fungi. The strain of phytase has been developed of researches to improve the thermostability and pH profile. The pyhtase used in monogastric animals in recent animal products industry commonly. Nowadays, an Aspergillus niger PhyA mutant phytase and an Escherichia coli AppA2 mutant phytase have been used more often in animal feed industry. Yi and Kornegay (1996) reported that there were varying levels of phytase activity in different species and Augspurger et al. (2003) found that different species had different responses to phytase supplementation. Considering phytase improved phosphorous absorption by change the intestinal pH, there is necessary to take a view of the relationship between phytase and GI microbiota.

1.4 Terminal restriction fragments length Polymorphisms

Terminal restriction fragments length Polymorphisms (T-RPLP) are a recently introduced PCR-based tool for studying microbial community structure and dynamics. This molecular microbiology technique method is produce a pattern or profile of nucleic acids amplified from a sample and that pattern reflects the microbial community structure, providing investigators with a large amount of easily analyzed data on microbial community structure. After PCR, restriction enzyme digestion and gel electrophoresis or database matching steps, you can get a general view of the microbiological composition of your samples.

CHAPTER 2

Objectives

2. Objectives

The aim of this study was to get the relationship between feed additives and intestinal health. Supplementing pigs with different diet ingredients that can have an effect on the GI tract microbiota is a common strategy to affect pig's health. In this study, in order to detect the non-antibiotic anti-microbial effect of nutritional additives, all the three trials show three different resource additives using variety techniques.

This study consists of three independent trials, all the feed additives used in this study are non-antibiotic aim to increase the intestinal anti-microbial effect. Trial 1's purpose is to optimize the plant polyphenols complex formulation based on in vitro antioxidant and microbial modulation ability. Trial 2 was to determine effects of supplemental Escherichia coli AppA2 and Aspergillus niger PhyA phytases on composition changes of the four major intestinal bacteria in weanling pigs. The aim of trial 3 was to evaluate the efficacy and tolerance of Sodium Benzoate in weaned piglets fed mixed diet. The effect of Na-benzoate on the intestinal microflora in piglets was also investigated.

CHAPTER 3

Optimization of plant polyphenols complex based on in vitro antioxidant and microbial modulation ability

3. Optimization of plant polyphenols complex based on in vitro antioxidant and microbial modulation ability

3.1 Abstract

The purpose of this study was to optimize the plant polyphenols complex formulation based on in vitro antioxidant and microbial modulation ability. Firstly, the uniform design was employed to study the formula of plant polyphenols mixture. Apple, grape seed, green tea and olive leaf polyphenols were adopted with 6 different levels each and the experiment number amounted to fifteen. The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH*) scavenging capacity, antimicrobial activities including against E. coli, Staphylococcus aureus and Salmonella typhimurium (isolated from the diarrhea piglet) were determined. The stepwise regression method was used to deal with the determined values of different treatments. The results exhibited that it had the optimal effects when apply, grape seed, green tea and olive leaf polyphenols accounted for 16.5%, 27.5%, 30% and 2.5% respectively in the mixture. Secondly, in avoidance of possible growth inhibition effect against the beneficial bacterium in the intestine due to the superb antioxidant capacity of the plant polyphenols mixture, a series of Mannan inclusion was applied to determine the proper concentration of the adjuvant which would not negatively affect the growth of lactobacillus. The linear regression method was adopted and 19.5% was drawn as the appropriate concentration. Taken together, the optimal complex of plant polyphenols contained 16.5%, 27.5%, 30%, 2.5% of apply, grape seed, green tea and olive leaf polyphenols as well as 19.5% of Mannan based on the results obtained here. And subsequent validation test confirmed the positive effects of the optimal mixture with respect to antioxidant and microbial modulation ability. Our results indicated that it was feasible to optimize the plant polyphenols complex by using uniform design based on their in vitro activity.

3.2 Introduction

The Plant polyphenols is one of the most powerful groups of bioactive compounds with antimicrobial and antioxidant property, which represents a diverse group of compounds (Hammer et al. 1999; Heim et al. 2002). The

commonly available plant polyphenols involve green tea polyphenol, grape polyphenol, apple polyphenol and olive oil polyphenols, etc. Although some polyphenols are considered to be non-nutritive, interest in these compounds has arisen because of their possible beneficial effects on health. It has already been reported that polyphenols from olive leaf exert superb antimicrobial activity (Paul et al. 1997), tea polyphenols improve gut microflora balance in calves (Ishihara et al. 2001) and inhibit the proliferation of Chlamydia (Yamazaki et al. 2003), apple polyphenol extracts prevent damage to human gastric epithelial cells (Graziani1et al. 2005), and polypheols from grape seeds could prevent oxidative damage to cellular DNA in vitro (Fan and lou, 2004). A considerable amount of evidence concerning the immunomodulatory function of plant polyphenols has also been found (Hughes, 2005). Furthermore, it is believed that the efficacy of the antimicrobial compound will not be compromised due to the development of pathogen resistance. The British Pharmacopoeia (1996 Edition) reports that microorganisms do not build resistance to benzyl alcohol, phenols, polyphenols, and similar products.

Uniform design is a novel efficient fractional factorial design, which was proposed by Professor Fang Kai-Tai and Professor Wang Yuan in 1980. It has been successfully used in various fields such as chemistry and chemical engineering, pharmaceutics, quality engineering, system engineering, survey design, computer sciences and natural sciences (Fang, 1980). The uniform design has been recognized as an important space-filling design by the international community. The uniformity and disperse rather than the orderliness and comparability would be taken into account in uniform design. And more information would be obtained due to increased degree of uniformity and separation with fewer and representative experimental treatments (zhang et al. 1993). Uniform arrangement is one of the robust designs that may reduce the experimental labor without compromising the experimental accuracy.

Polyphenol compound ranks fourthly in the components of plant, next to cellulose, hemicellulose and lignin. Abundance in nature makes it an ideal alternative for substitution of antibiotics used in animal feed. Application of plant extract in animal feed is a promising field of further research. Regardless of large number of studies on the properties of individual plant, specific data in the literature concerning their complex mixture on antimicrobial and antioxidant ability was scarce.

Therefore, the purpose of this study was to determine the optimal plant polyphenols complex according to the antioxidant and microbial modulation effects. The optimal plant polyphenols mixture would be firstly achieved by

using uniform design based on in vitro antioxidant capacity and antimicrobial activities again pathogens prevailing in piglet production. And subsequently, inclusion of appropriate concentration of mannan oligosaccharide in the mixture would be obtained based on the viability of lactobacillus in view of the possible inhibition on beneficial bacterial due to excellent antioxidant activity of polyphenols so as to optimize the plant polyphenols complex. The optimized formulation of the complex would be tested in the following piglet trial, and the results of this study could serve as the base for plant polyphenols application in swine nutrition.

3.3 Materials and methods

Plant polyphenols used in this study were abundant in resources and available in the market, i.e. apple, grape seed, green tea and olive leaf polyphenols. All of them were purchased from famously normal enterprises with export certificate. And the main ingredient of the four plant polyphenols was listed in table 1.

Table 1 Information of the plant polyphenols used in the study

Plant polyphenol	Price	Main active ingredients and contents	Producing area
Apple polyphenol	1500RMB/kg	Total polyphenol (80.15%), Phlorizin(5.09%）, Chlorogenic acid（17.10%）,Anthocyanin B2（12.86%）	Tianjin Jianfeng Natural Product Co., Ltd. China
Grape seed polyphenol	800 RMB/kg	Anthocyanin（99.47%）, Anthocyanin compound（65.19%）, Anthocyanin B2（3.39%）	Tianjin Jianfeng Natural Product Co., Ltd. China
Green tea polyphenol	426RMB/kg	Total catechins (75.34%, EGCg(47.47%) Caffeine (5.81%)	Taiyo Green powder Co., Ltd. China
Olive leaf polyphenol	380RMB/kg	Oleuropein (21.28%)	NingboSino-Taipio Herbal Science Co., Ltd. China

Six levels of each plant polyphenol were designed and allotted into 15 treatments using uniform design (table 2). The specific concentration of the plant plolyphenols in the treatments was shown in table 3. The carrier used in the study was silicon dioxide.

Table 2 Uniform design table for U15* (15 4)

.Treatment	1	2	3	4
1	1	3	2	5
2	2	3	5	4
3	3	3	1	1
4	4	1	6	3
5	5	2	3	3
6	6	6	2	5
7	1	4	3	2
8	2	2	3	4
9	3	5	5	6
10	4	6	1	3
11	5	2	2	6
12	6	4	4	2
13	1	5	6	1
14	2	1	4	1
15	3	1	1	2

Table 3 Uniform design of the plant polyphenols material (%)

No.	X1(Apple)	X2（Grape seed）	X3（Green tea）	X4（Olive leaf）	Carrier
1	1 (0.5)	3 (12.5)	2 (10)	5 (32.5)	44.5
2	2 (3.5)	3 (12.5)	5 (25)	4 (25)	34
3	3 (6.5)	3 (12.5)	1 (5)	1 (2.5)	73.5
4	4 (9.5)	1 (2.5)	6 (30)	3 (17.5)	40.5
5	5 (12.5)	2 (7.5)	3 (15)	3 (17.5)	47.5
6	6 (15.5)	6 (27.5)	2 (10)	5 (32.5)	14.5
7	1 (0.5)	4 (17.5)	3 (15)	2 (10)	57
8	2 (3.5)	2 (7.5)	3 (15)	4 (25)	49
9	3 (6.5)	5 (22.5)	5 (25)	6 (40)	6
10	4 (9.5)	6 (27.5)	1 (5)	3 (17.5)	40.5
11	5 (12.5)	2 (7.5)	2 (10)	6 (40)	30
12	6 (15.5)	4 (17.5)	4 (20)	2 (10)	37
13	1 (0.5)	5 (22.5)	6 (30)	1 (2.5)	44.5
14	2 (3.5)	1 (2.5)	4 (20)	1 (2.5)	71.5
15	3 (6.5)	1 (2.5)	1 (5)	2 (10)	76

3.3.1 Preparation of the plant polyphenol mixture

In For the pant polyphenol mixture solution preparation, 200 milligram of solute (plant polyphenols and carrier) was added into 200 milliliter 0.85% saline water. The undissolved carrier served to the balance of solute in the liquid.

3.3.2 DPPH scavenging ratio

Same The radical 2,2-diphenyl-1-picrylhydrazyl (DPPH*) is a stable radical. Antioxidant capacity was determined using DPPH* scavenging ratio. In its radical form, DPPH* absorbs at 515 nm but when reduced by an antioxidant (AH likephenolic compounds) or a radical species (R*), absorption ceases:

$$DPPH^* + AH \rightarrow DPPH\text{-}H + A^*$$

$$DPPH^* + R^* \rightarrow DPPH\text{-}R$$

Therefore, the absorption at 515 nm was proportional to the amount of residual DPPH*.

For the determination of DPPH scavenging ratio, 2 milliliter of plant polyphenols mixture was added to same amount of a 6×105 mol methanol DPPH* solution. The absorbance was determined at 515 nm at 37℃ after 30 minutes. The sample blank tube contained 2 mL plant polyphenol mixture and 2 mL of methanol. The blank control tube contained 2 mL methanol in addition to DPPH solution. And the DPPH scavenging ratio was calculated according to the following formula:

DPPH scavenging ratio (%)=[1-Asample-Asample blank]/Acontrol]×100%

DPPH was purchased from sigma-Aldrich.

3.3.3 Microbial modulation test

3.3.3.1Antimicrobial activity against pathogen

Three pathogen bacteria including E. coli, Staphylococcus aureus and Salmonella typhoid, purchased from Chinese Institute of Veterinary Drug Control were selected to determine the antimicrobial activity of plant polyphenols. The latter two bacteria were separated from the diarrhoea piglets' intestine in the field.

For the pathogen growth inhibition test, 100 microlitre of 5×104 cfu/mL bacterial solution was added into 1milliliter polyphenol mixture solution and reacted at 37℃ for 4 hours. Afterward, 50 microlitre of the diluted mixture was laid on the 9 cm round flat containing the corresponding culture medium and cultured for the certain hours for the count of bacterium. And the diluted

bacteria solution with saline water correspondingly served as the control. The pathogen growth inhibition ratio was calculated followed the formula:

Growth inhibition ratio= (count in control plate-count in mixture plate)/count in control plate×100%

E. coli and Staphylococcus aureus populations were assessed by using agar after being incubated at 37°C for 24 h. Salmonella typhoid populations were determined using SS Culture medium after being incubated at 37°C for 36 h.

3.3.3.2 Viability of lactobacillus

After the acquisition of sub-optimal plant polyphenols mixture based on the antioxidant capacity and antimicrobial activities against the pathogens, a series of plant polyphenols complex were prepared by addition of different levels of mannan oligosaccharide purchased from All-tech company (seen detail in results part). The modulation effect of the plant polyphenols complex on Lactobacillus viability was determined. Lactobacillus population were assessed after plated on the Man, Rogosa, and Sharp agar and incubated anaerobically at 37°C for 48 h. The viability ratio= count in mixture plate/count in control plate×100%

3.3.4 Statistical analysis

Data obtained from antioxidant capacity and antimicrobial activity against pathogen bacteria were analyzed with Uniform Design software (5.0, Chinese Uniform Design association, 2005), and stepwise regression was applied to get the optimal solution. Data for the lactobacillus viability was analyzed by using linear regression model.

3.4 Results

3.4.1 DPPH scavenging ration and pathogen inhibition effects

Table 4 Results of antioxidant and antimicrobial activity of plant polyphenols mixture (%)

No.	X1	X2	X3	X4	Scavenging ratio of DPPH	Inhibition ratio of *E coli*	Inhibition ratio of *Staphylococcus aureus*	Inhibition ratio of *Salmonella typhoid*
1	0.5	12.5	10	32.5	90.1925	76.1905	23.0337	69.5581
2	3.5	12.5	25	25	88.449	61.5646	2.2472	98.527
3	6.5	12.5	5	2.5	89.2481	92.517	26.6854	26.0229

4	9.5	2.5	30	17.5	87.5772	59.5238	33.427	88.5434
5	12.5	7.5	15	17.5	86.7054	48.2993	-3.652	66.4484
6	15.5	27.5	10	32.5	81.6201	77.2109	21.9101	99.3453
7	0.5	17.5	15	10	92.372	26.1905	14.0449	-15.057
8	3.5	7.5	15	25	90.1925	68.0272	19.6629	24.7136
9	6.5	22.5	25	40	92.7352	81.6327	32.5843	100.00
10	9.5	27.5	5	17.5	89.9019	65.8163	20.7865	74.4681
11	12.5	7.5	10	40	87.2866	66.8367	3.6517	97.7087
12	15.5	17.5	20	10	90.2652	74.1497	23.0337	96.8903
13	0.5	22.5	30	2.5	90.2652	76.5306	39.3258	86.9067
14	3.5	2.5	20	2.5	89.3207	22.619	22.7528	-28.478
15	6.5	2.5	5	10	88.7396	7.9932	30.618	9.329

Note: the 3 bacteria were purchased from Chinese Institute of Veterinary Drug Control, and the E. coli and the Salmonella typhoid were isolated from the intestine of diarrhoea piglets.
Data showed the results of incubation bacteria with plant polyphenols mixture for 4 h.

Table 4 showed the data of DPPH scavenging ratio and the pathogen inhibition effects of plant polyphenols mixtures in the treatments. The mixtures exhibited excellent antioxidant capacity with more than 80 percent of DPPH scavenging ratio. Inhibition effects of plant polyphenol mixture against E.coli and Salmonella typhoid was steady and effective except that two treatments had the stimulating effects on Salmonella typhoid growth. And five treatments with more than ninety five percent inhibition ratio against Salmonella typhoid revealed that the remarkable repression effect were definitive. The antimicrobial activity against Staphylococcus aureus was somewhat less effective and unstable compared to the other two pathogens, and the highest inhibition ratio was less than 40 percent.

By fitting apple, grape seed, green tea and olive leaf polyphenols concentration as independent variable X1, X2, X3 and X4, and DPPH *scavenging ratio, growth inhibition ratio of E. coli, staphylococcus aureus and salmonella typhoid as dependant variable Y1, Y2, Y3 and Y4, the regression equations were obtained and showed in table 5.

Table 5 The regression analysis results for the plant polyphenols mixtures

Parameter	Regression equations	R^2	P
DPPH scavenging ratio	$Y_1=95.57685-0.7787X_1-0.8635X_2+0.3830X_3-0.3931X_4+0.0342X_1^2+0.0187X_1X_3-0.0201X_1X_4+0.0536X_2^2$	0.9872	0.0112
***E.coli* inhibition ratio**	$Y_2=-78.3946+12.3691X_1+16.3303X_2-2.8866X_3+3.0860X_4-1.2963X_1^2+0.3108X_1X_2+0.3088X_1X_3+0.0694X_1X_4$	0.8859	0.0215
Staphylococcus aureus	$Y_3=114.4262-8.1315X_1-4.3529X_2-5.5973X_3-0.9564X_4+0.1497X_1X_2+0.3048X_1X_3+0.0281X_1X_4+0.1211X_2^2$	0.9523	0.0703

inhibition ratio			
***Salmonella typhoid* inhibition ratio**	$Y4=-86.1382+13.5263X_1+5.9826X_2-5.9197X_3+3.1482X_4+0.6240X_1^2-0.0920X_1X_2-0.6815X_1X_3-0.3731X_1X_4$	0.9996	0.0339

In ANOVA analysis and significance test showed that the equation correlation coefficients were nearly or above than 0.9 and probability less than 0.05 except the probability in equation for Staphylococcus aureus. The results indicated that the regression relationship of antioxidant capacity and Salmonella inhibition equation were significant and effective, and furthermore, the parameters adopted here was feasible for the optimization of plant polyphenols mixture.

Based on the integrated analysis from the regression equations, it could be obtained that when apple, grape seed, green tea and olive leaf polyphenols accounted for 16.5%, 27.5%, 30% and 2.5% in the complex mixture, the combined mixture exhibited superb antioxidant capacity and pathogen bacteria inhibition effects. The optimal solution was listed in Table 6.

Table 6 Integrated optimal solution for superb antioxidant and antimicrobial activities from the regression equations

	X1	X2	X3	X4	Ymax/Ymin	ki	Optimal solution Yi
Y1	0.5	2.5	26.1914	40	115.07694	1	104.5217
Y2	8.7188	19.0039	5	40	2.49753	1	126.9742
Y3	16.5	27.5	30	2.5	139.9972	1	139.9972
Y4	0.5	11.1426	30	40	2.8404	1	70.7449
Integrated optimal solution	16.5	27.5	30	2.5			

When different factors within antioxidant capacity, antimicrobial activity and cost were paid diverse attention, the optimal solutions for the plant polyphenos mixtures were different and their compositions varied a lot. Table 7 showed the optimal solutions for plant polyphenols mixture composition according to the factors concerned.

The solution showed in table 6 was the optimal result based on antioxidant and antimicrobial efficacy. Taking the factor of cost into account besides antioxidant and antimicrobial effects, the optimal mixture would consisted of the minimum amounts of apple, grape seed and green tea polyphenols as well as the maximum amounts of olive leaf polyphenols. If only antioxidant effect and cost were

considered, the mixture contained minimum concentrations of apple, grape seed plant polyphenols, and medium concentration of green tea and maximum concentration of olive leaf.

Table 7 Optimal solutions for combinations for plant polyphenols mixture Based on different factors concerned

		Apple	Grape seed	Green tea	Olive leaf
Mixture A	Antioxidant (1) Inhibition of *E coli* (1) Inhibition of *Staphylococcus aureus* (1) Inhibiton of *Salmonella* (1)	16.5	27.5	30	2.5
Mixture B	Antioxidant (1) Inhibition of *E coli* (1) Inhibition of *Staphylococcus aureus* (1) Inhibiton of *Salmonella* (1) Price or cost (-1)	0.5	2.5	5	40
Mixture C	Antioxidant (1) Price or cost (-1)	0.5	2.5	21.2	40

3.4.2 Validation test of the calculated sub-optimal mixture

In order to verify the effects of the calculated sub-optimal plant polyphenols mixture, a validation experiment was conducted by using mixture A and B as observation object. The results in table 8 showed that the both mixtures had similarly strong antioxidant capacity with more than 91 percent of DPPH scavenging ratio. Mixture A exhibited moderate inhibition ratio against E.coli and remarkable inhibition ratio against salmonella typhoid, whereas in mixture B only lower inhibition effects against the two pathogenic bacteria were found. And the inhibition effect of staphylococcus aureus was uncertain and even negligible in the two mixtures. Since the validation test was performed with different batch of operation afterwards, the results confirmed that the plant polyphenol mixture A possessed the superb antioxidant capacity and antimicrobial activity against Salmonella typhoid.

Table 8 Antioxidant and antimicrobial effects of the calculated mixture A and B

Item	DPPH scavenging ratio (%)	Inhibition ratio of *E.coli*	Inhibition ratio of *Staphylococcus aureus*	Inhibition ratio of *Salmonella typhoid*
Mixture A	91.57	53.90	1.87	98.72
Mixture B	91.54	11.20	-2.25	16.66

3.4.3 Further optimization of the plant complex by inclusion of probiotic

In view of the superb antioxidant capacity of the mixture, it was hypothesized that the mixture may affect the growth of beneficial bacteria. Thus, seriate concentrations of mana-oligosaccharide (MOS) were included in the sub-optimal mixture of plant polyphenols with the aim to determine the suitable dose of MOS that would not negatively affect the growth of lactic acid bacterium based on the viability examination.

Table 9 Lactobacillus viability (%) of different inclusion of MOS in plant polyphenols mixture

MOS addition ratio (%)	Lactobacillus viability (%)	DPPH	E.coli	*Staph. aureus*	Salmonella typhoid
0	80.5183	91.57	53.90	1.87	98.72
2.5	86.8633	91.50	52.85	1.96	97.55
5	87.9357	91.55	51.95	1.79	98.19
7.5	84.361	91.46	53.76	1.85	97.88
10	90.4379	91.52	53.48	1.91	98.46
12.5	95.4424	91.54	53.69	1.77	98.64

Table 9 showed the result of lactobacillus viability treated by the mixtures supplemented with seriate concentrations of MOS. In accordance with the hypothesis, the mixture void of MOS inhibited the growth of lactobacillus by about 20 percent of the viability. With the increase of MOS supplementation dose in the mixture, the viability of lactobacillus increased steadily and arrived to 95.44 percent when MOS level amounted to 12.5%. The linear regression analysis was adopted to predict the growth curve of lactobacillus (figure 1) and the R square of the equation came to 0.73. By fitting 100% into the linear regression equation, the appropriate concentration of MOS in the mixture was obtained as 19.5% in the mixture.

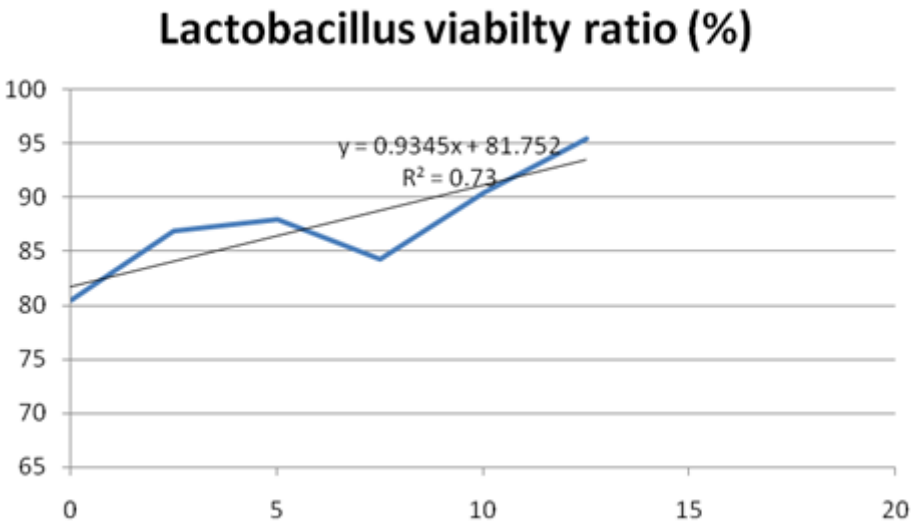

Figure 2 Growth curve of the lactobacillus with the increasing dose of MOS in the mixture

Therefore, the optimal plant polyphenos mixture with excellent antioxidant capacity, antimicrobial activity against E.coli and Salmonella typhoid without compromising the viability of lactobacillus would contain 16.5%, 27.5%, 30%, 2.5%, 19.5% and 4% of apple, grape seed, green tea, olive leaf polyphenols, MOS and carrier respectively.

It is expected that the optimized plant polyphenol mixture would exert promising effect on modulation the function of gastrointestinal tract and immunity of the early weaning piglet. And the in vivo animal trial concerning the investigation of the prevention effects against salmonella adhesion in the gastrointestinal of weaned piglets is pending to conduct.

3.5 Discussion

The antioxidant and antimicrobial activity of plant extracts and oil has been recognized for many years. Data from different studies all demonstrated plant extracts possess divergent extent or spectrum against different pathogens. In our study, we combined the four plant polyphenols to obtain a superb mixture with the expectation that synergistic action existing within them irrespective of the solo activity of the single plant extract.

In summary, this study confirms that the plant polyphenols possess in vitro antimicrobial activity and antioxidative property, and the optimized solution of plant polyphenols mixture was obtained by using the uniform design based on

the antimicrobial and antioxidant activity. In vivo studies may be required to confirm the validity of the optimized plant polyphenols obtained herein.

Previous studies reported the assimilation of plant polyphenols (Hollman et al. 2000) and their ability to confer antioxidant protection in vivo. Oral administration of grape flavonoids has been shown to confer antioxidant protection (O'Byrne et al. 2002), which is confirmed in this study.

Stable radicals such as the 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical had been used to evaluate the antioxidant activities of flavonoids in vitro in this study. These plant phenolics have gained increasing interest because of their numerous properties and biological effects such as free radical scavenging, modulation of enzymatic activities, and inhibition of cellular proliferation, as well as their potential utility as antibiotic, antiallergic, and antiinflammatory agents.

Several studies have reported that specific polyphenols scavenge superoxide radicals and hydroxyl radicals, reduce lipid peroxyl radicals, and inhibit lipid peroxidation. Plants produce thousands of phenolic compounds as secondary metabolits. Rich sources include tea, wine, fruits and vegetables contain considerable polyphenolic products. Evidence is accumulating that plant polyphenols play a role in antioxidant capacity. Polyphenols are effective antioxidant in a wide range of chemical oxidation system (Duthie and Crozier, 2000). The DPPH-scavenging activities of apple polyphenols were 2-3 times better than those of antioxidant vitamins C and E (Lu and Foo,2000).

Plant polyphenols used in this study were very effective scavengers of DPPH* in vitro.

Tea catechins and polyphenols are effective scavengers of reactive oxygen species in vitro and may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities.

EGCG and EGC , the main ingredients of tea polyphenols, have superior electron donors to vitamin E and inferior to vitamin C, indicating that the reactivity of acting as hydrogen or electron donors in tea plyphenols is among that of vitamine E and C (Jovanavic et al. 1996).

Antioxidant activity of oleuropein is mainly due to the hydroxytyrosol moiety in its structure. This ability to scavenging the ABTS_+ radical cation in comparison with hydroxytyrosol is lower due to the increased molecular weight;

The most abundant compound in OL is oleuropein, It is significative that OL had an antioxidant activity higher than vitamin C and E, due to the synergy between flavonoids, oleuropeosides and substituted phenols.

Tea is a source of epigallocatechin gallate, in green tea, and theaflavin and in black tea. The most abundant phenolic compounds isolated from grape seed are catechins, epicatechin, procyanidin, and some dimers and trimers.

The data obtained reveal that the extracts are free radical inhibitors and primary antioxidants that react with free radicals.

Jayaprakasha et al. (2003) reported that Gram-positive bacteria were completely inhibited at 850–1000 ppm, while Gram-negative bacteria were inhibited at 1250–1500 ppm concentration of grape seed extracts.

3.6 References

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CHAPTER 4

Supplemental dietary phytase alters gut microbiota of weanling pigs

4. Supplemental dietary phytase and stromtium alters gut microbiota of weanling pigs

4.1 Abstract

Past phytase research has been largely focused on nutritional values of the enzyme, and has not explored its impact on gut microbiota of animals. The objective of this study was to determine effects of supplemental Escherichia coli AppA2, Aspergillus niger PhyA phytases and strontium on composition changes of the four major intestinal bacteria in weanling pigs. Two piglets trial were conducted to investigate the gut microflora composition change when the diet added different kinds of phytase enzymes and strontium by using terminal restriction fragments length polymorphism (T-RFLP) analysis of 16s rRNA genes. The first trial diets were supplied with phytase enzyme and strontium. 32 piglets were divided into 4 groups feed by basal diet, basal diet with a bacterial phytase at 3,500 units/kg (OptiPhos, JBS United, Sheridan, IN), basal diet with strontium at 4% (Synergy-1, Orafti, Tienen, Belgium) and phytase/strontium of the same dosage together added into basal diet for five weeks. In the second trial, a total of 24 crossbreds (3-week old, Yorkshire- Landrace-Hampshire crossbred) were allotted to three groups (n = 8) and fed a cornsoybean- mean basal diet (BD, supplemented with 0.35% inorganic phosphorus), the BD plus 3,500 units of AppA2/kg (Optiphos, JBS United, Sheridan, IN), or the BD plus 3,500 units of PhyA/kg (Natuphos, BASF, Florham Park, NJ) for six weeks. The 16s rRNA gene were isolated after PCR amplification with universal primer sets of total genomic DNA extracted from each sample of ileum and colon adherent from each piglets. In this study, we focus on 4 kinds of microflora lactobacilli, enterococcus group, clostridium group and streptococci, which are confirmed main intestinal bacteria of monogastric animals. Results show that compare with nasal diet, bacterial phytase at 3,500 units/kg (OptiPhos, JBS United, Sheridan, IN) can increase the bifidobactera , streptococci spp and also E.coli/Samonella group in ileum ($p < 0.05$) and phytase increase the E.coli/Samonella group in colon significantly ($p < 0.01$). in the second trial, both of the two phytase enzymes increase the amount of bifidobacteria significantly in ileum adherent ($p < 0.01$) but the E.coli/Samonella group shows a significante decrease after treated by the two kinds of phytase enzymes also in ileum adherent samples ($p < 0.01$). PhyA also decrease the clostridium spp and E.coli/Samonella in colon adherent ($p < 0.05$). In conclusion, AppA2 and PhyA seem more functional to change the gut microflora composition.

4.2 Introduction

Phytase is added in animal feeds to improve phosphorus nutrition and reduce phosphorus excretion of swine and poultry in areas of intensive animal agriculture. The commercial use research way of phytase has been explored for a long time. Phytase is produced by plants, bacteria, and fungi. The strain of phytase has been developed of researches to improve the thermostability and pH profile. The pyhtase used in monogastric animals in recent animal products industry commonly.

Nowadays, an Aspergillus niger PhyA mutant phytase and an Escherichia coli AppA2 mutant phytase have been used more often in animal feed industry. Yi and Kornegay (1996) reported that there were varying levels of phytase activity in different species and Augspurger et al. (2003) found that different species had different responses to phytase supplementation. Considering phytase improved phosphorous absorption by change the intestinal pH, there is necessary to take a view of the relationship between phytase and GI microbiota.

A few experiments (17,18) have shown the potential benefits of dietary phytase conducted to optimize growth and production responses of pigs, data on bone responses of pigs from these studies offered limited implications for intestinal health issues.

Strontium has been used as a marker for calcium metabolism, previous study showed that the piglets feed by strontium had a significance improvement on performance and bone mineral content. The nutrition absorption has a close relationship with intestinal microbiological status. Therefore, there is necessary to get some information about how the phytase and strontium absorption affect the GI microbiota composition

Terminal restriction fragments length Polymorphisms (T-RPLP) is popular used on study the microbial community structure nowadays. This molecular cultivation-independent techniques are now being used increasingly as routine tools for the analysis of the complex intestinal ecosystem, which providing investigators with a large amount of easily analyzed data on microbial community structure. These ribosomal sequences are present in all cellular life forms and are highly conserved, but also contain variable regions, which provide the possibility to discriminate among bacterial phylotypes. After PCR, restriction enzyme digestion and gel electrophoresis or database matching steps, you can get a general view of the microbiological composition of your samples.

4.3 Materials and methods

This trial contains two experiments: Experiment 1 used two kind of phytase as feed additives and experiment 2 used both phytase and strontium as feed additives by adequate dose.

4.3.1 Animals, diets and treatments

4.3.1.1 Experiment 1:

All animal protocols and housing was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee. Two phytases (Escherichia coli AppA2 and Aspergillus niger PhyA phytases) were selected using information from previous studies (Kim, Weaver, & Lei, 2008; Zhang & Lei, 2007). A total of 24 crossbreds (3-week old, Yorkshire- Landrace-Hampshire crossbred) were allotted to three groups (n = 8) and fed a corn soybean- mean basal diet (BD, supplemented with 0.35% inorganic phosphorus), the BD plus 3,500 units of AppA2/kg (Optiphos, JBS United, Sheridan, IN), or the BD plus 3,500 units of PhyA/kg (Natuphos, BASF, Florham Park, NJ) for six weeks.

4.3.1.2 Experiment 2:

Our protocol was approved by the Institutional Animal Care and Use Committee of Cornell University. All pigs used in the study were weanling crossbreds (Landrace- Hampshire-Duroc) selected from the Cornell University Swine Farm. Pigs were weaned at 4 wk of age, and allotted into treatment groups based on body weight, litter, and sex. The pigs were divided into 4 groups (n=8) and were fed BD, BD with phytase (2000 U/kg), BD with Sr (50 mg/kg) or BD (one piglet lost in phytase group at the end of the trial.) with phytase (2000 U/kg) and Sr (50 mg/kg). The phytase used in this experiment was Escherichia coli AppA2 (OptiPhos, JBS United). After the actual activity was analyzed (25), the phytase enzyme was added to the diets at feed mixing. Strontium was added to the diet in the form of SrCO3 (Alfa Aesar). Pigs were penned in an environmentally controlled barn (20 – 25℃; 12h light: 12h dark cycle), and were allowed free access to feed and water.

Table4.1 Composition of the basal diet 1

Ingredient	*g/kg*
Corn, grain	657.50
Soybean meal, 48% CP	280.00
Spray-dried plasma protein	15.00
Corn oil	10.00
L-Lysine ·HCl	1.00
Vitamin/mineral premix[1]	2.00
Dicalcium phosphate	13.50
Limestone	10.50
MgO_4	0.50
Salt	5.00
Tylan[2]	5.00
Total	1000.00
Nutritional values	
ME,[3] MJ/kg	14.1
Crude protein,[3] %	20.0
Ca, total, %	0.81
P, total, %	0.66
P, available, %	0.33
Total Ca:P	1.24

[1] Vitamin and mineral premix/kg diet: retinyl palmitate, 1650μg; cholecalciferol, 27.5 μg; dl-α-tocopheryl acetate, 16.08 μg; menadione, 0.73 mg; d-biotin, 26 μg; choline chloride, 66 mg; niacin, 26.4 mg; Ca-D-panthothenate, 17.6 mg; riboflavin, 4.4 mg; CuSO4·5H2O, 6 mg; C2H8N22HI, ethylene diamine dihydroiodide, 0.14 mg; MnO, 4 mg; Na2SeO3, 0.3 mg; ZnO, 100 mg.
2 Antibiotic additive (Tylan 10) contains tylosin (as tylosin phosphate) at 22 g/kg (Elanco).
2 Calculated based on NRC (24).

4.3.2 Sample collection

The piglets were euthanized at the end of the trials. The lumen adherent samples were collected immediately after sacrifice from ileum and mid-colon from each piglet. All the samples of the gut adherent were collected by detergent wash. Adherent bacteria are released by gentle agitation of tubes for 30 minutes. Centrifuge lumen and adherent wash tubes at 11000-12000 rpm for 5 minutes. Carefully remove supernatant and resuspend pellets in 5 mL BHIB/20% glycerol Samples were kept at -80°C until further processing.

The recipe for 1L detergent wash is as follows:

Combine 50 mL 0.5M Tris-HCl (pH 7.6), 100 mL 10% (v/v) TX-100 in sterile MQ-H2O, 100 mL 10% (w/v) SDS in sterile MQ-H2O, 100 mL 10% (w/v) SDC in sterile MQ-H2O, and 20 mL 0.5M EDTA (pH 7.6). Make up to

800 mL with sterile MQ-H2O. Adjust the pH to 7.6 with NaOH. Make up to 1 L with sterile MQ-H2O.

The recipe for 1L BHIB/20% (v/v) glycerol is as follows:

Dissolve 37g BHIB in 600 mL MQ-H2O. Add 200 mL glycerol. Make up to 1000 mL with MQ-H2O. Sterilized by autoclaving at 121°C.

4.3.3 DNA extraction

The DNA of each sample was isolated by a phenol-chloroform –isoamyl alcohol method modified from Yu et al. [Improved extraction of PCR-quality community DNA from digesta and fecal samples. Zhongtang Yu and Mark Morrison The Ohio State University, Columbus, OH, USA. BioTechniques 36:808-812 (May 2004)] by the addition of a lysis step. Cell lysis was accomplished by incubating (24h for sand and 1h for cultures at 28 degree) 200μl of pelleted cells in 200ul of lysis buffer consisting of 100nM NaCl, 10 mM Tris HCl, 25 mM disodium EDTA and 700U/L lysozyme.

DNA isolated from all sample types was visualized on a 1.0% agarose gel in pH 8.25 TBE buffer (89.2mM Tris-base, 88.9mM boric acid, 2.47mM disodium EDTA) stained with ethidium bromide and quantified by comparison with DNA standards.

4.3.4 T-RFLP analysis.

PCR: primers: Fluorescently labelled forward primer (6FAM-27f; 6-FAM = 6 carboxyfluorescein; Sequence: AGA GTT TGA TCM TGG CTC AG)

Unlabelled reverse primer (1492r; Sequence: TAC GGY TAC CTT GTT ACG ACT)

Reactions were carried out in5 PCR tubes of 50 μl with 1*buffer (GoTaq DNA Polymerase buffer, Promega, USA), 0.6 mM dNTP, 8 μg/mL bovine serum albulmin, 2mM MgCl2, and 1U of Taq DNA polymerase.(GoTaq DNA Polymerase, Promega, USA) Reaction temperatures and cycling for fecal and sand samples were: 94°C for 2min, 35 cycles of 94°C for 1 min, 48.5°C for 30s, 72°C for 2min, followed by 72°C for 10min. Products were visualized on 1.0% agarose gels and qualified as above.

Primer removal and amplicon concentration: do the purification using Promega Wizard Kit according to the instruction. The concentrated was washed twice and

resuspended in sterile deionized water. Resuspension volumes were varied to equalize amplicon concentrations.

4.3.5 Amplicon digestion

Digestion reactions contained 0.1 μg of labeled DNA and were incubated for 12h at 37°C. The 15-20 μl reactions contained 1.0 U/L of either Hha I or Bsh 1236I (Bio Tech, USA) in the manufacturer's recommended reaction buffers.
Digested samples washing and concentration: 96-well plate (Promega, USA), centrifuges 800g 15min.

4.3.6 Restriction Enzyme Digest Clean-up

Conducted this step using BioEdge Performa DTR Ultra 96-well plate. Follow manufacturer's protocol except will need to speed vac samples first to reduce volume to about 20 μl to use the 96-well column plate (Performa DTR).
After elution from 96-well plate, speed vac samples.

4.3.7 DNA solubilization and sample delivery:

Add 15 μL Hi Di Formamide (Applied Biosystems MicroAmp, USA) to all wells in the plate with sample. Pipet up and down once or twice to mix samples. Allow plate to sit on ice for about 10 minutes to allow DNA to solubilize.

Prepare a reaction mix containing the Liz 600 bp (Applied Biosystems MicroAmp, USA) size standard.

Use the multipetter to add 5 μL Liz reaction mix into each sample well.

Once all the wells have been filled with the necessary liquids, reseal the plate with a fresh piece of parafilm to prevent liquid evaporation. Place in a container for delivery. Cover plate with foil to keep out any light. Immediately deliver plate to the DNA sequencing facility for analysis using ABI 373 sequencer.

4.3.8 Statistical analysis.

TRFs dara were performed by software Peak Scanner (v1.0): Following the normalization and image analysis steps of the software. Export the data do the database comparison and significance analysis.

Database:

1. http://mica.ibest.uidaho.edu/ MiCA: Microbial Community Analysis: 2. The T-RFLP Phylogenetic Assignment Tool (PAT)https://secure.limnology.wisc.edu/trflp/index.jsp.

Peak data were analyzed using the one-way ANOVA General Linear Models procedure of SAS, version 6.12 (SAS Institute). Statistical significance was set at $P<0.05$.

4.4 Results and Discussion

4.4.1 PCR results:

4.4.1.1 Experiment 1

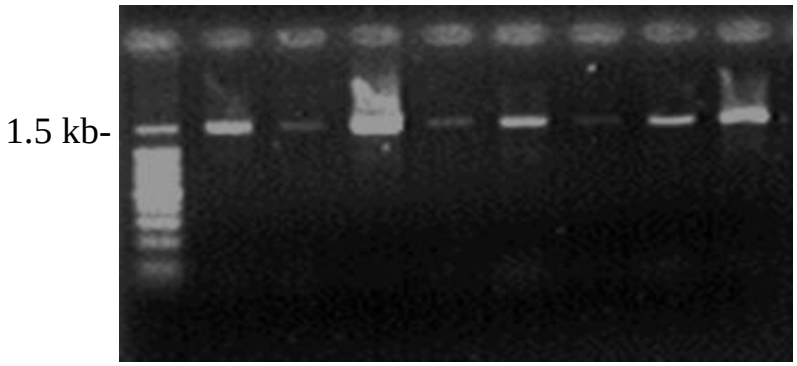


Figure 4.1 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of BD diet

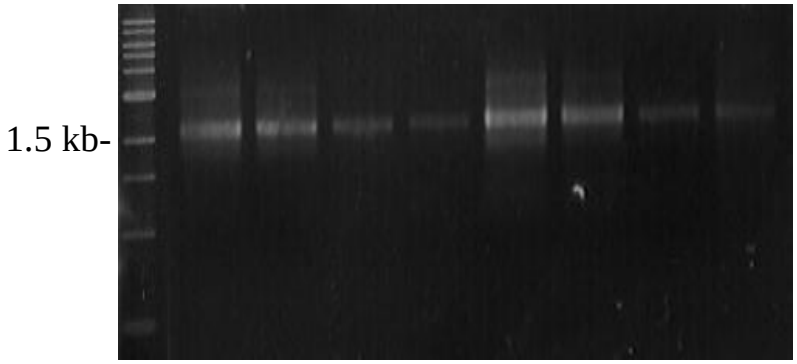


Figure 4.2 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of treatment 1(BD+ AppA2/kg).

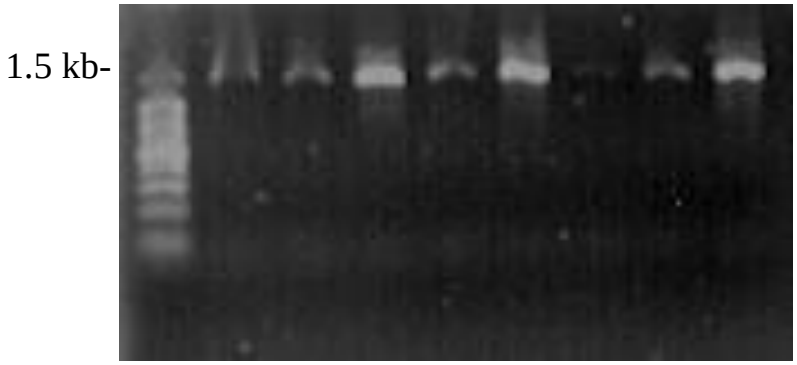


Figure 4.3 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of treatment 2(BD+ PhyA/kg).

4.4.1.2 Experiment 2

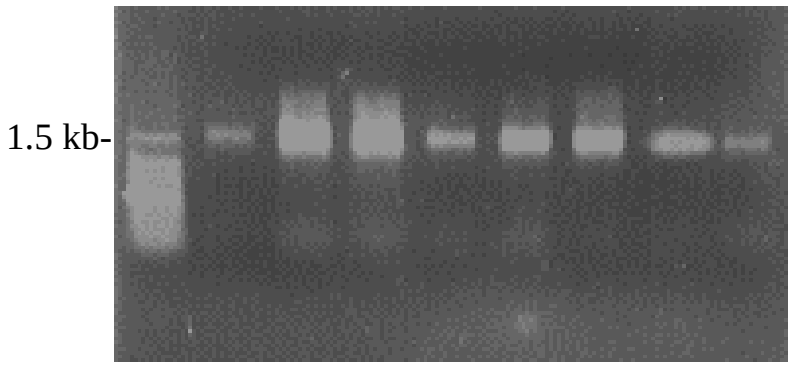


Figure 4.4 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of BD group.

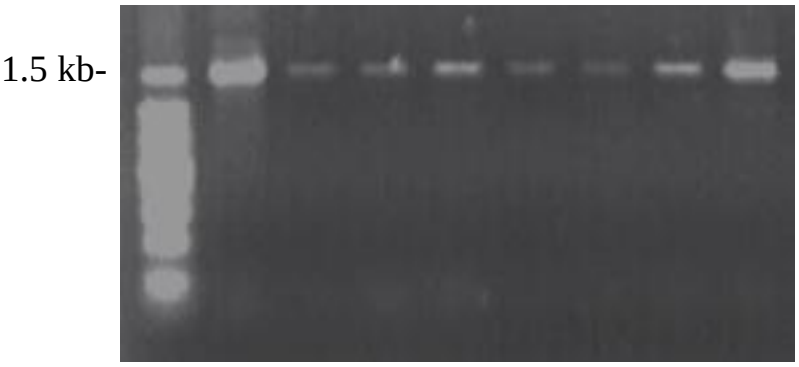


Figure 4.5 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of treatment 1 (BD+Phy).

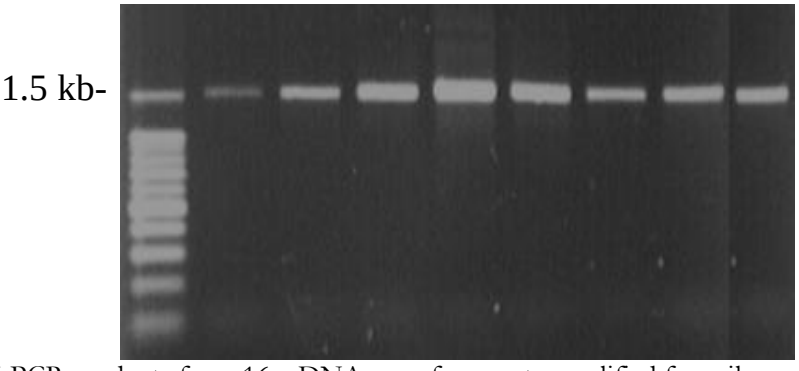


Figure 4.6 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of treatment 2 (BD+Sr).

1.5 kb-

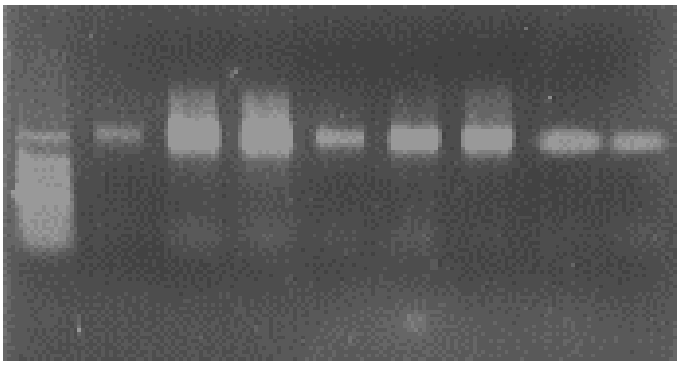
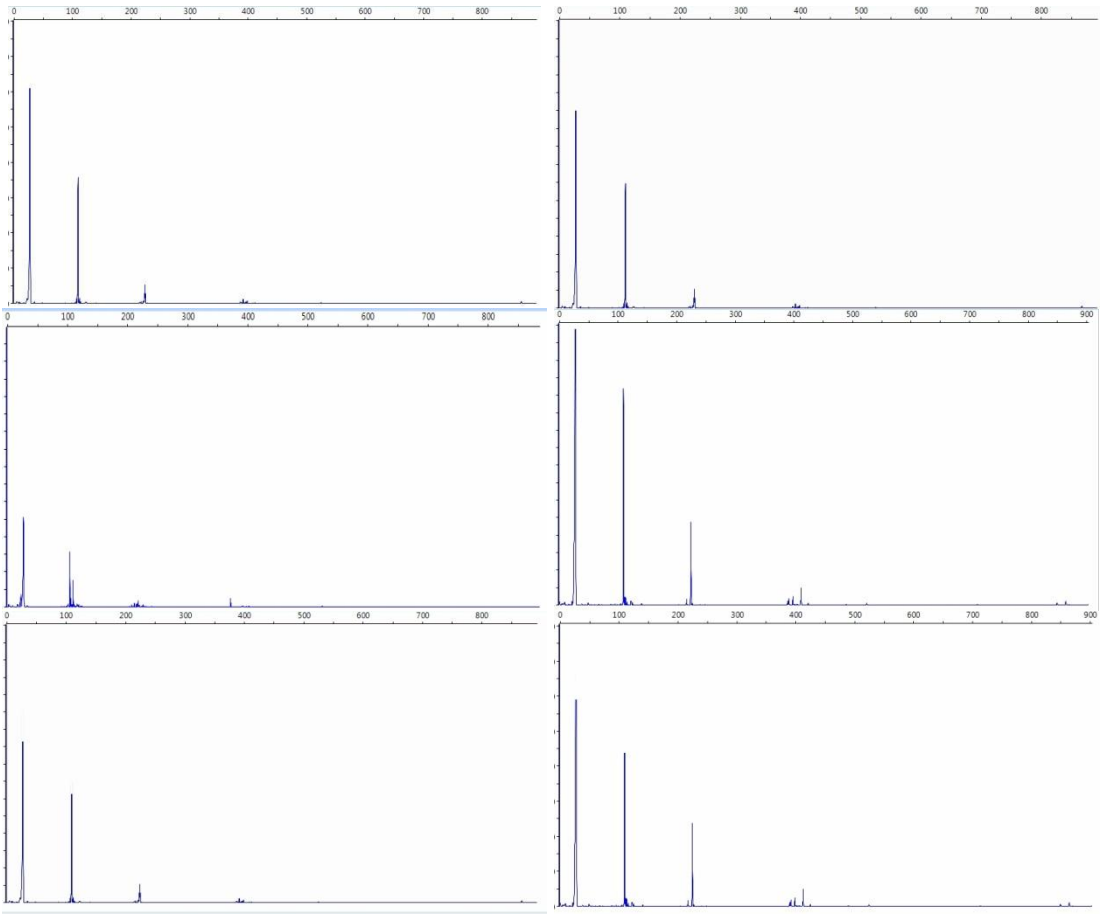


Figure 4.7 PCR products from 16s rDNA gene fragments amplified from ileum adherent samples of treatment 3 (BD+Phy+Sr).

4.4.2 TRFLP results:

4.4.2.1 experiment 1-Ileum adherent digested by Bsh 1236I



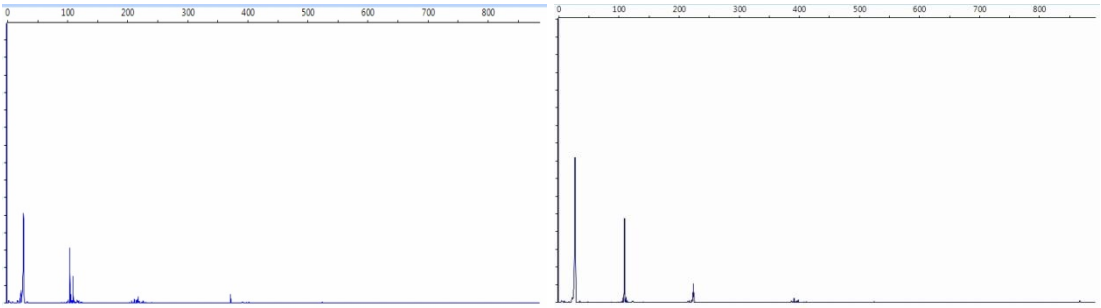


Figure 4.8 ileum adherent samples of BD group.

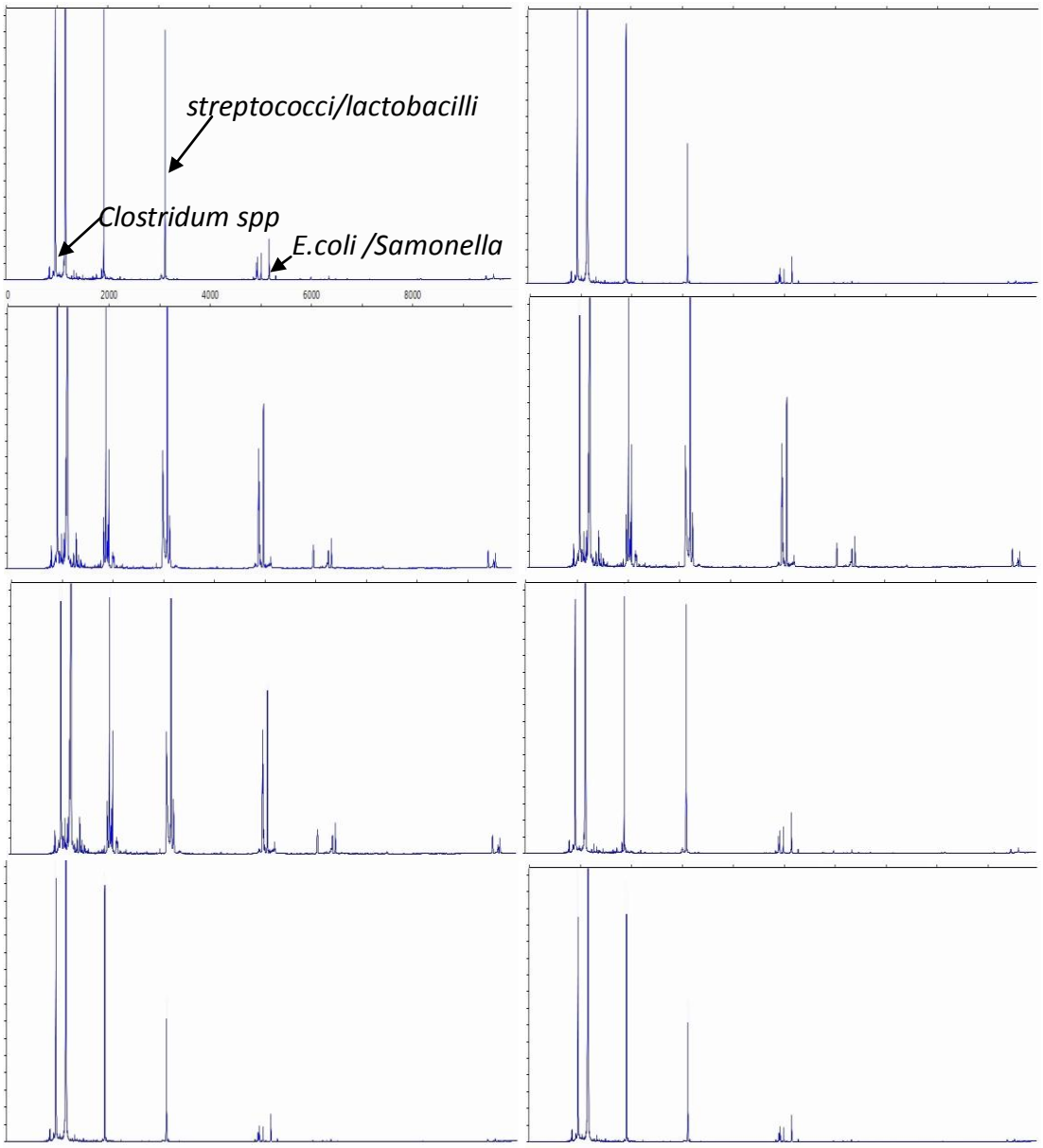


Figure 4.9 ileum adherent samples of treatment 1(BD+ AppA2/kg).

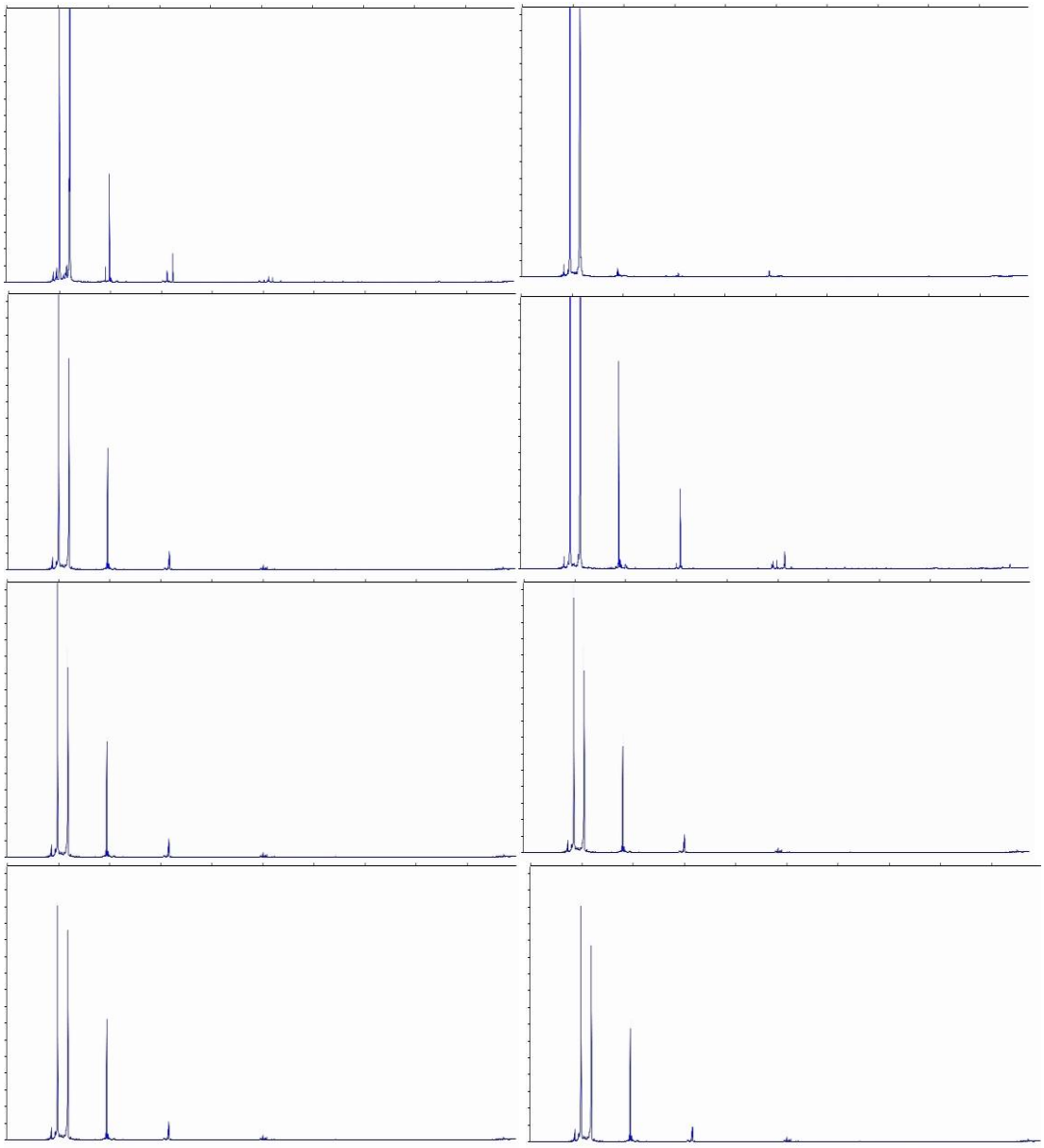


Figure 4.10 Ileum adherent samples of treatment 1(BD+ PhyA/kg).

4.4.2.2 Experiment 1-Ileum adherent digested by Hha I

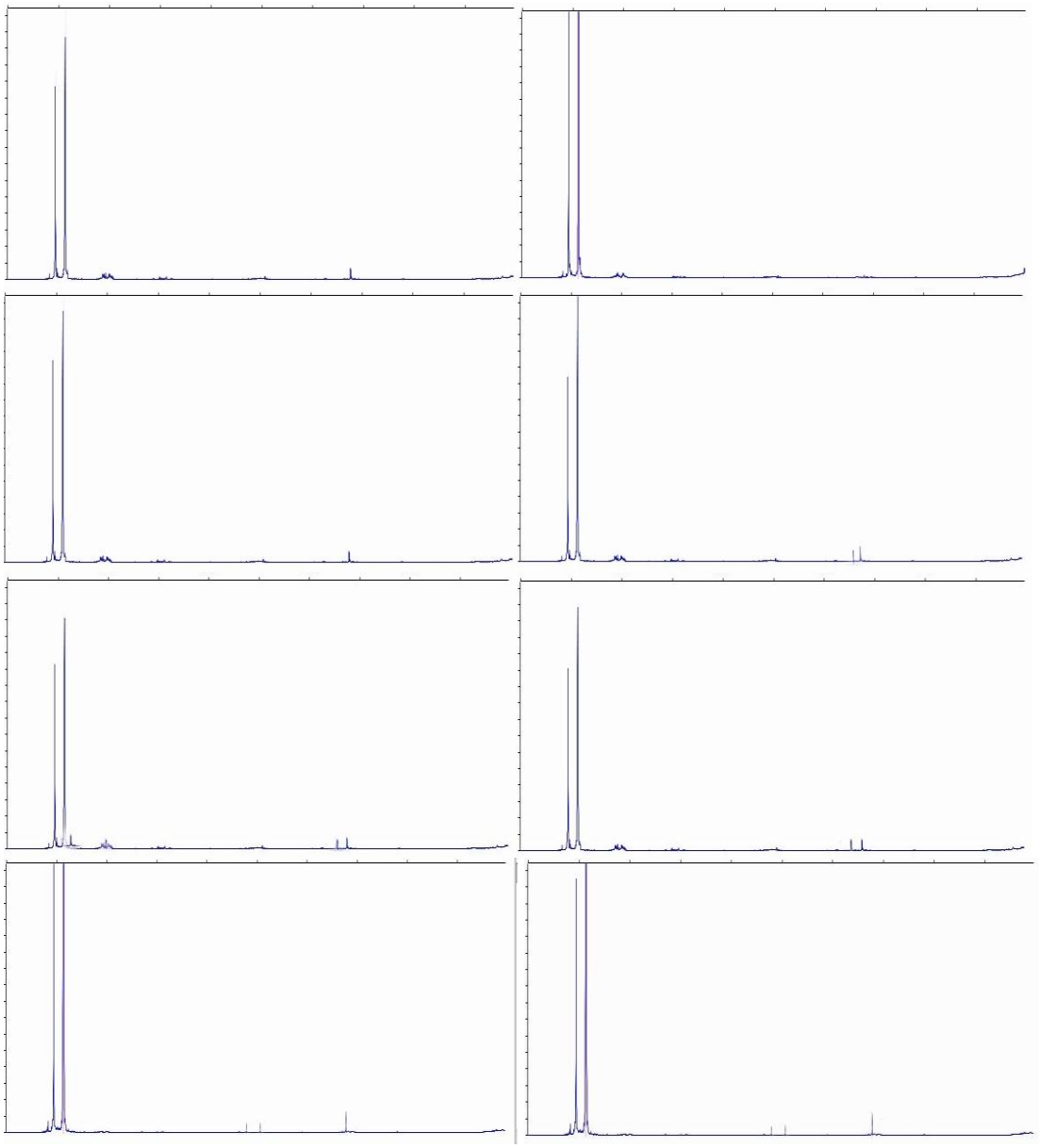


Figure 4.11 Ileum adherent samples of BD group

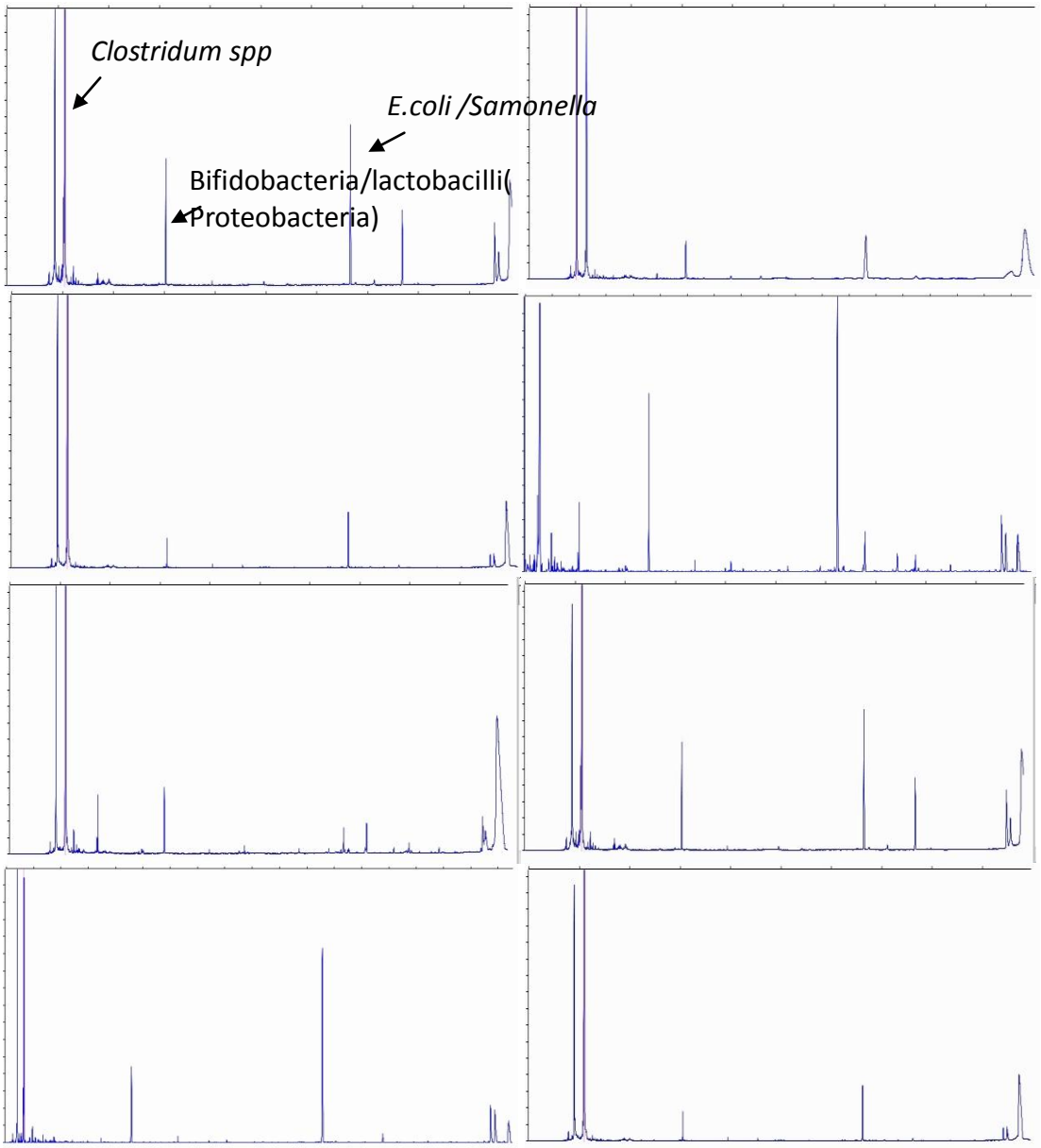


Figure 4.12 Ileum adherent samples of treatment 1(BD+ AppA2/kg).

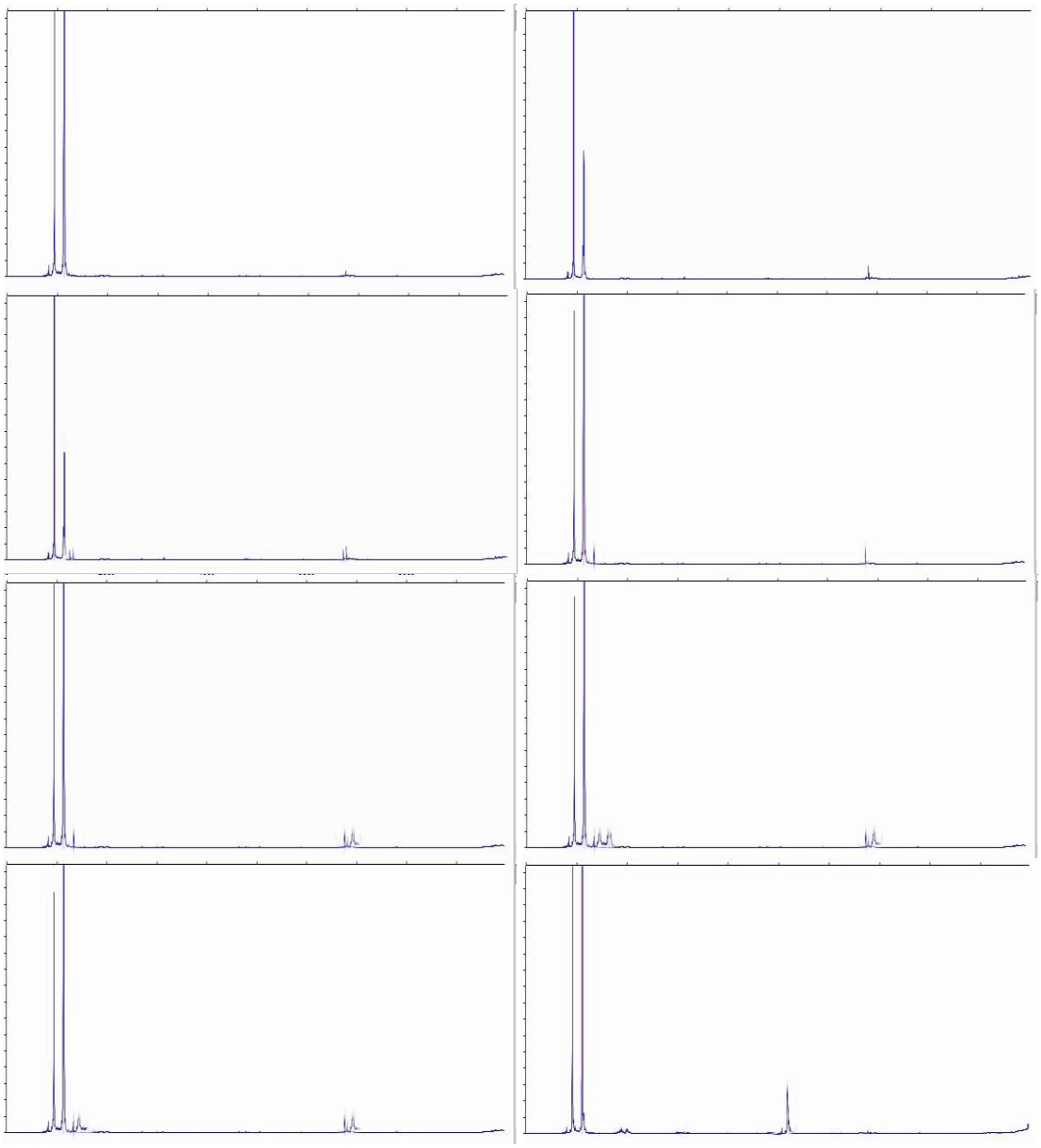


Figure 4.13 Ileum adherent samples of treatment 1(BD+ PhyA/kg).

4.4.2.3 Experiment 1-Mid-colon adherent digested by Bsh 1236I

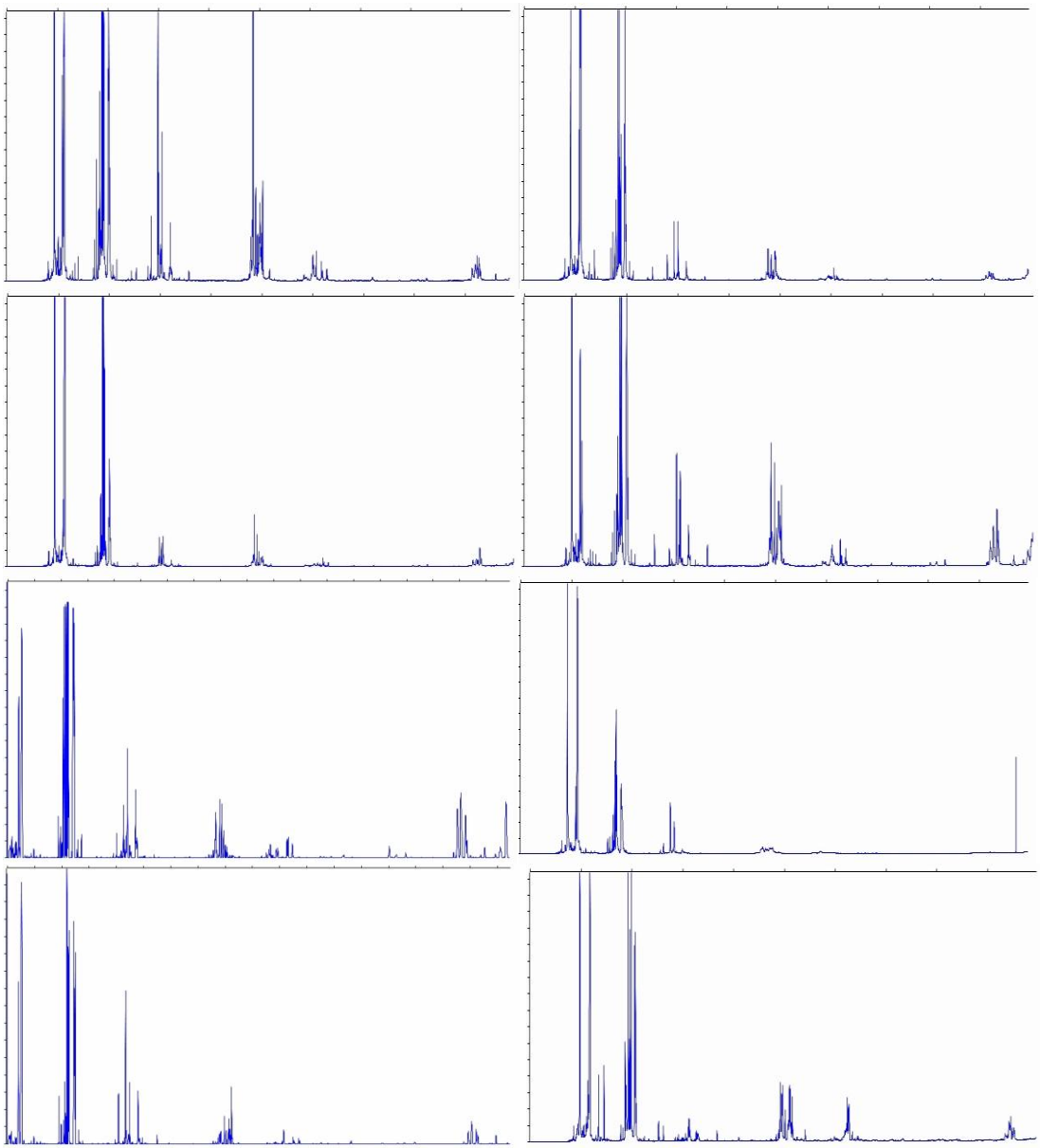


Figure 4.14 Mid-colon adherent samples of BD group

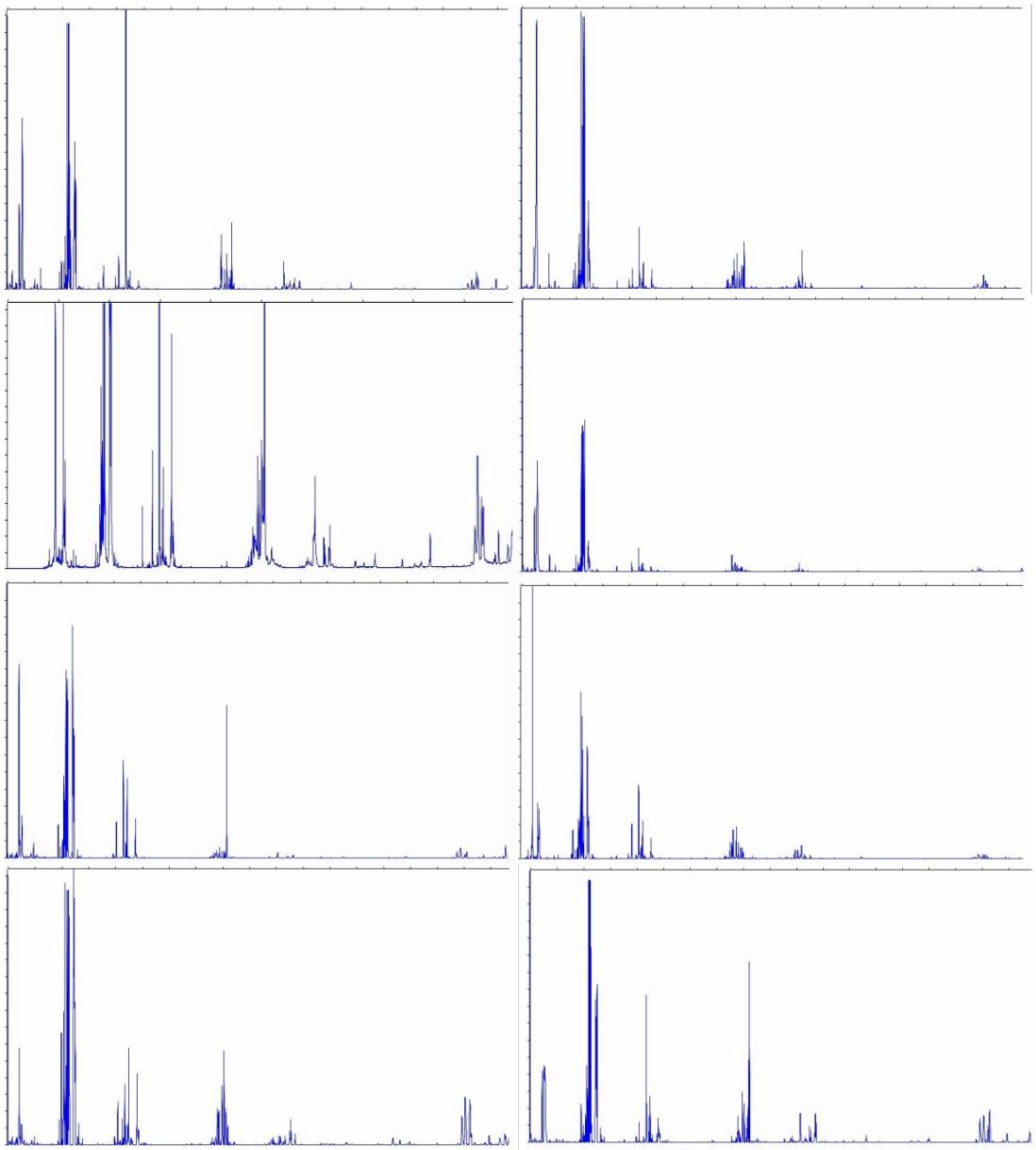


Figure 4.15 Mid-colon adherent samples of treatment 1(BD+ AppA2/kg).

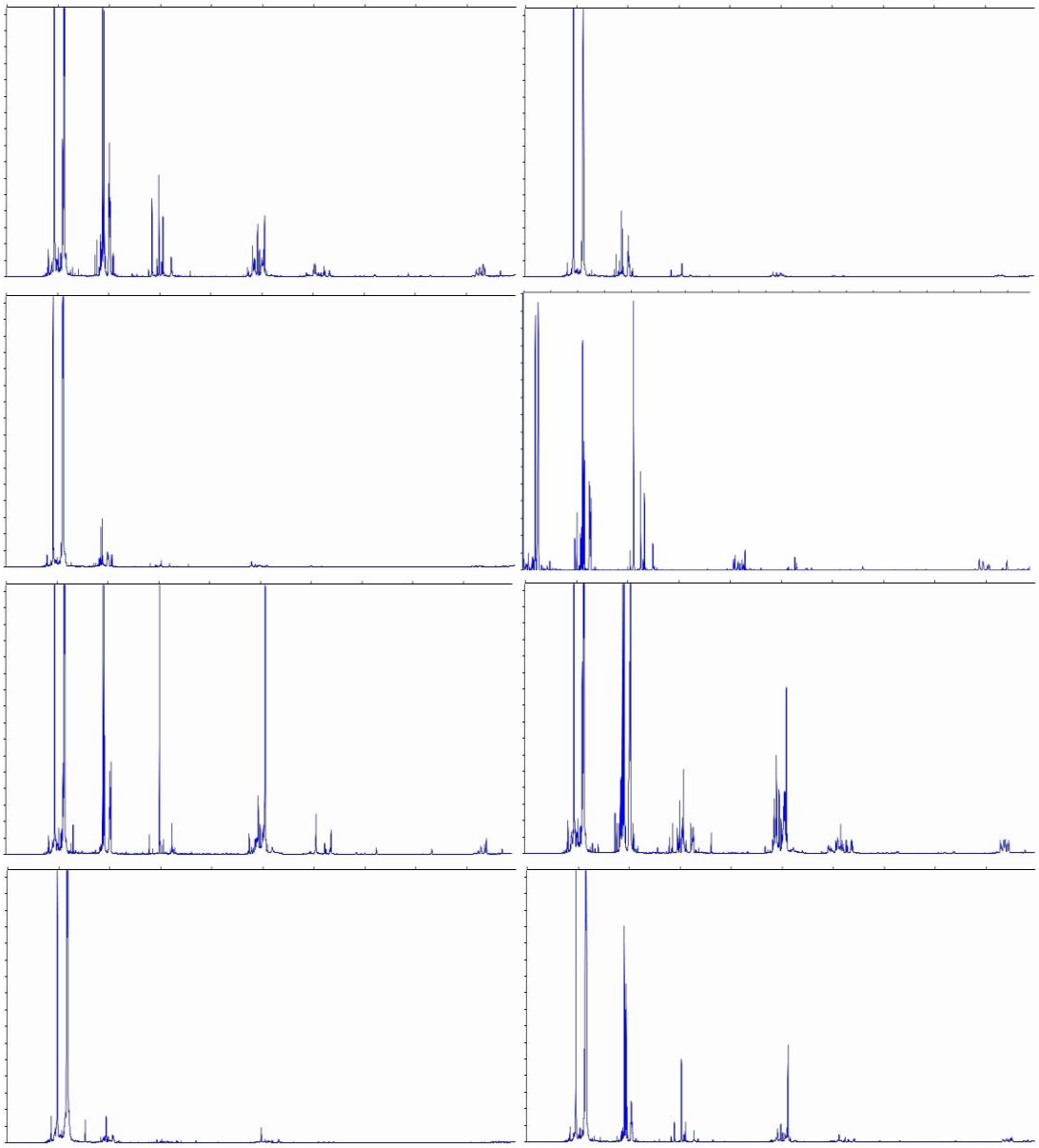


Figure 4.16 Mid-colon adherent samples of treatment 1(BD+ PhyA/kg).

4.4.2.4 Experiment 1-Mid-colon adherent digested by Hha I

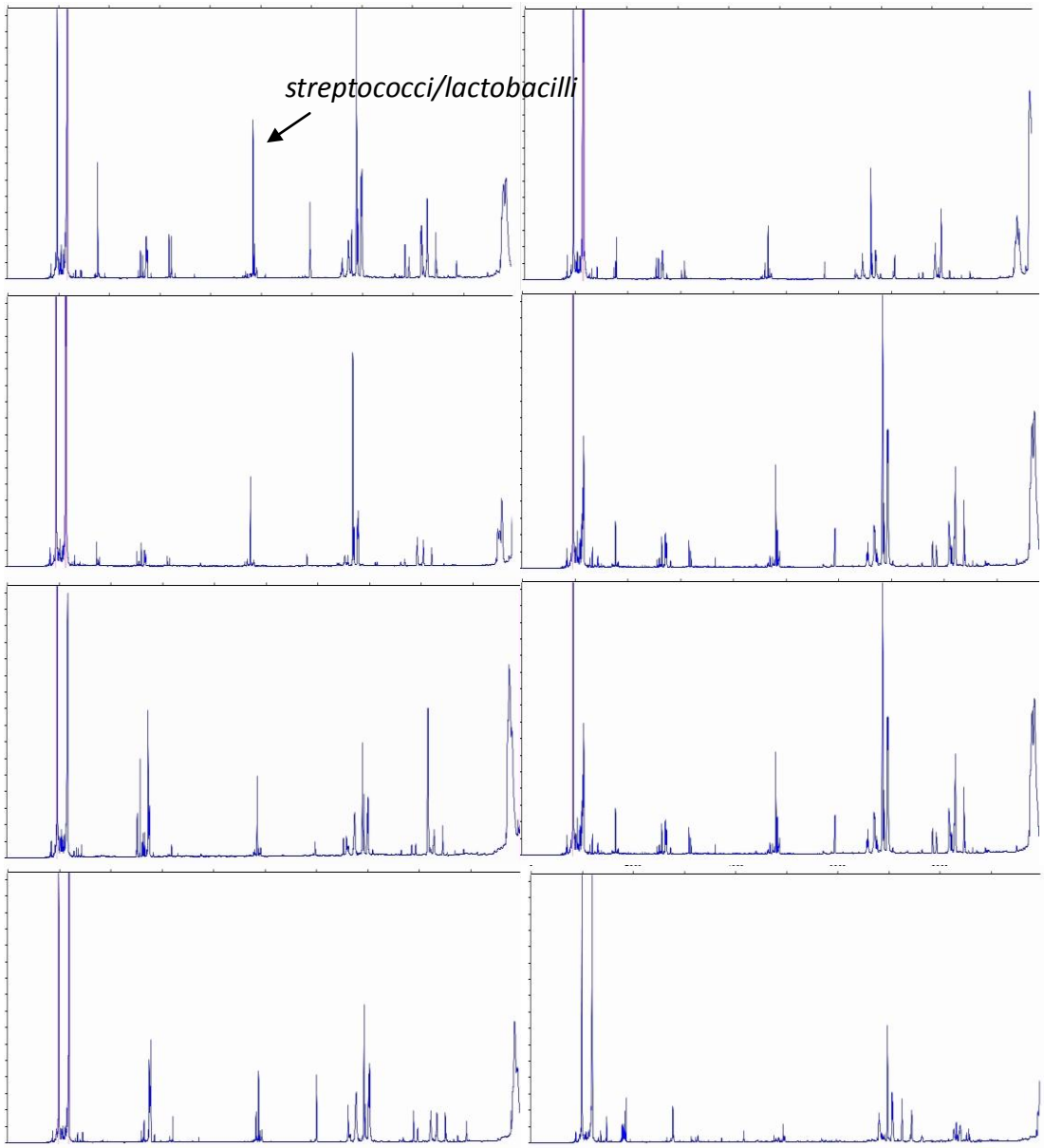


Figure 4.17 Mid-colon adherent samples of BD group.

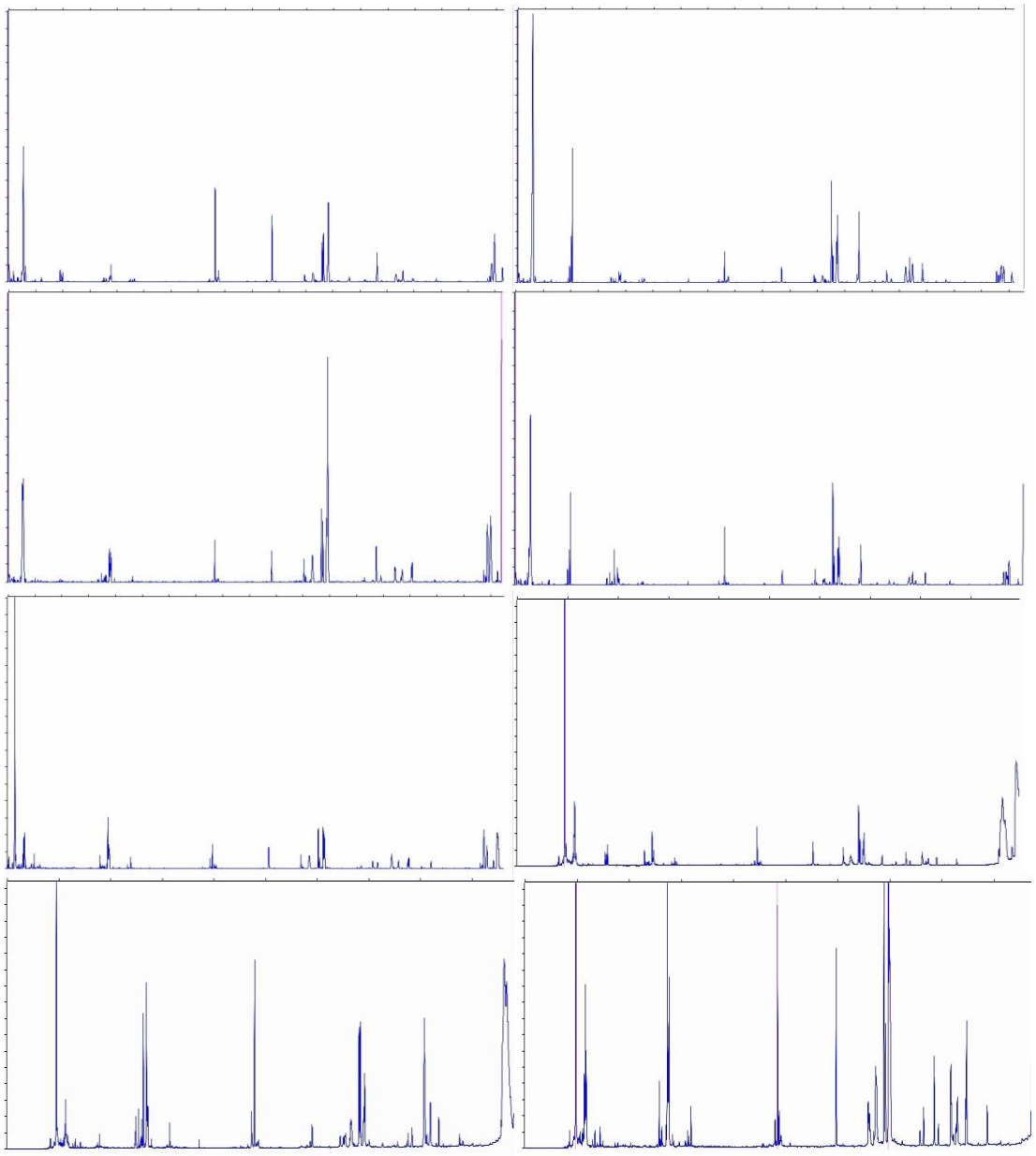


Figure 4.18 Mid-colon adherent samples of BD+AppA2/kg group.

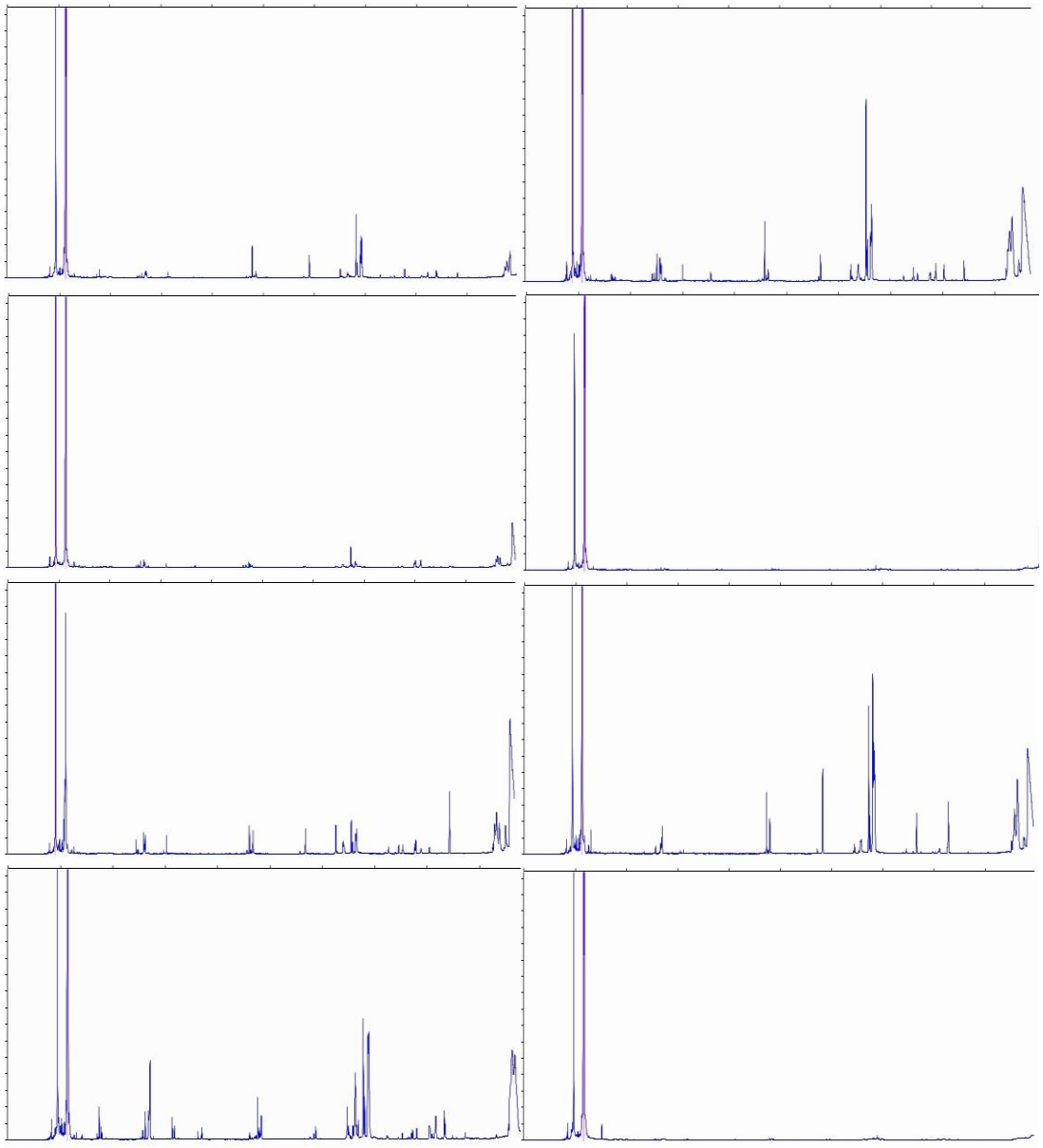


Figure 4.19 Mid-colon adherent samples of BD+PhyA/kg group.

4.4.2.5 Experiment 2-Ileum adherent digested by Bsh 1236I

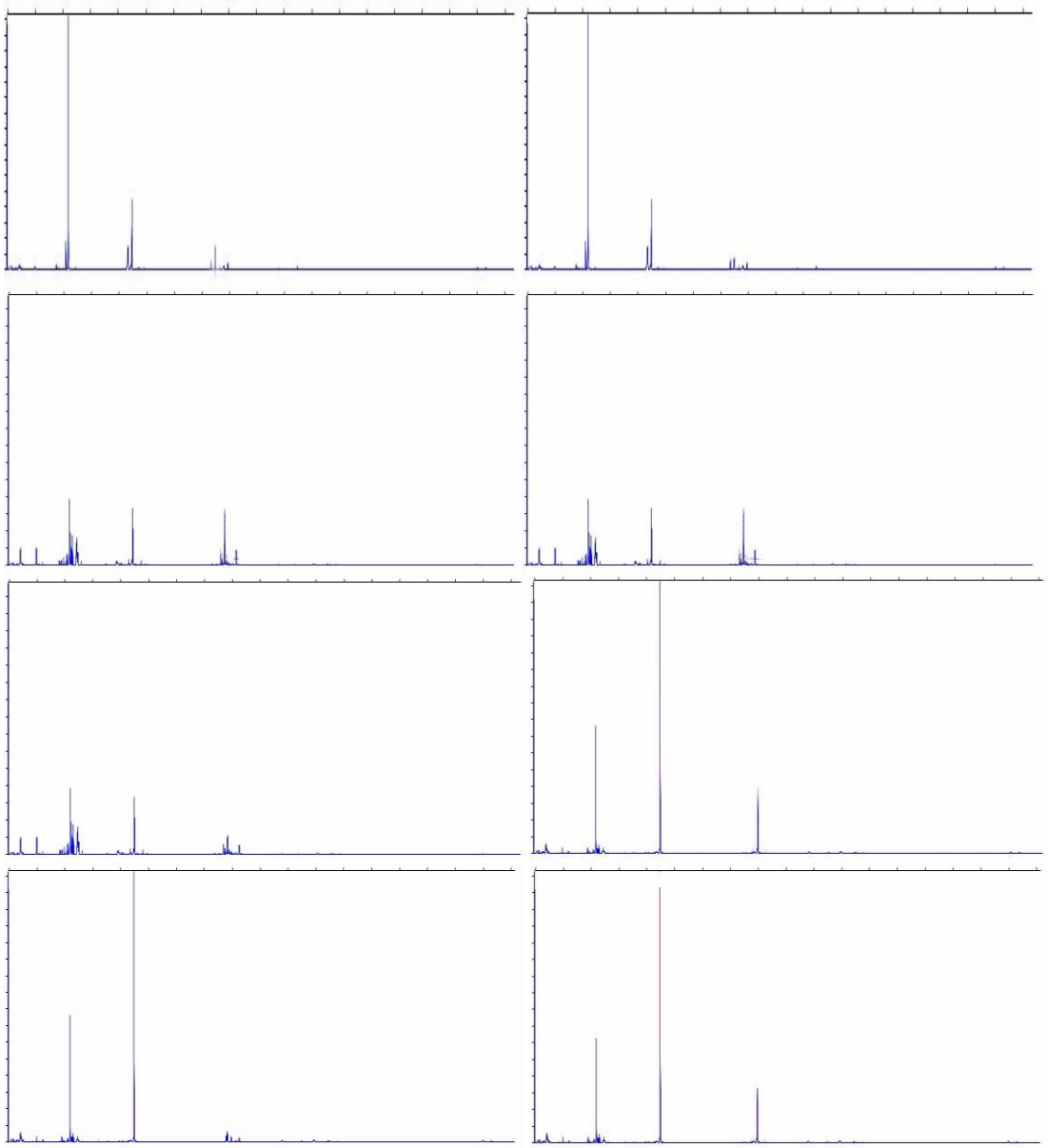


Figure 4.20 Ileum adherent samples of BD group.

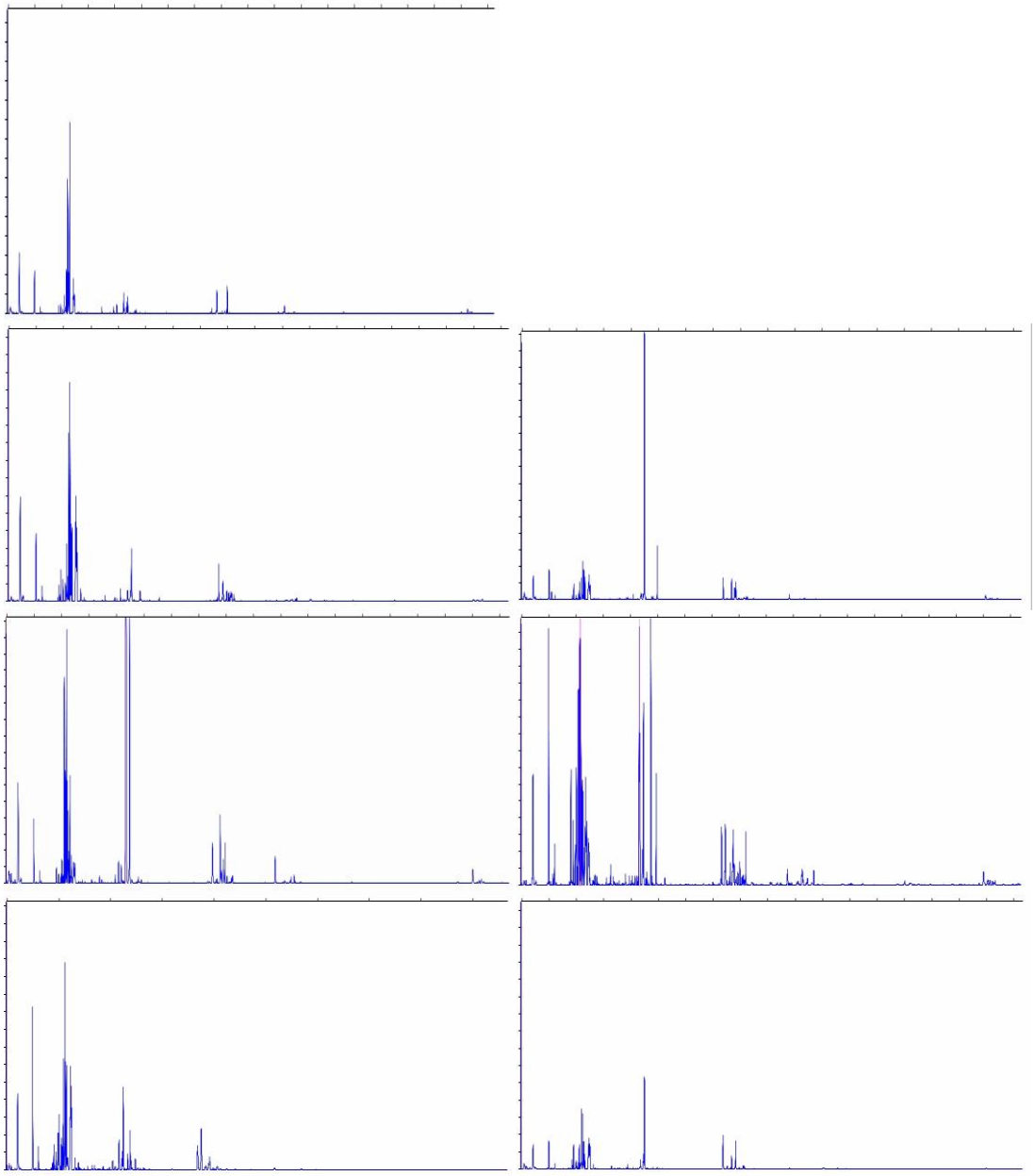


Figure 4.21 Ileum adherent samples of BD+Phy group.

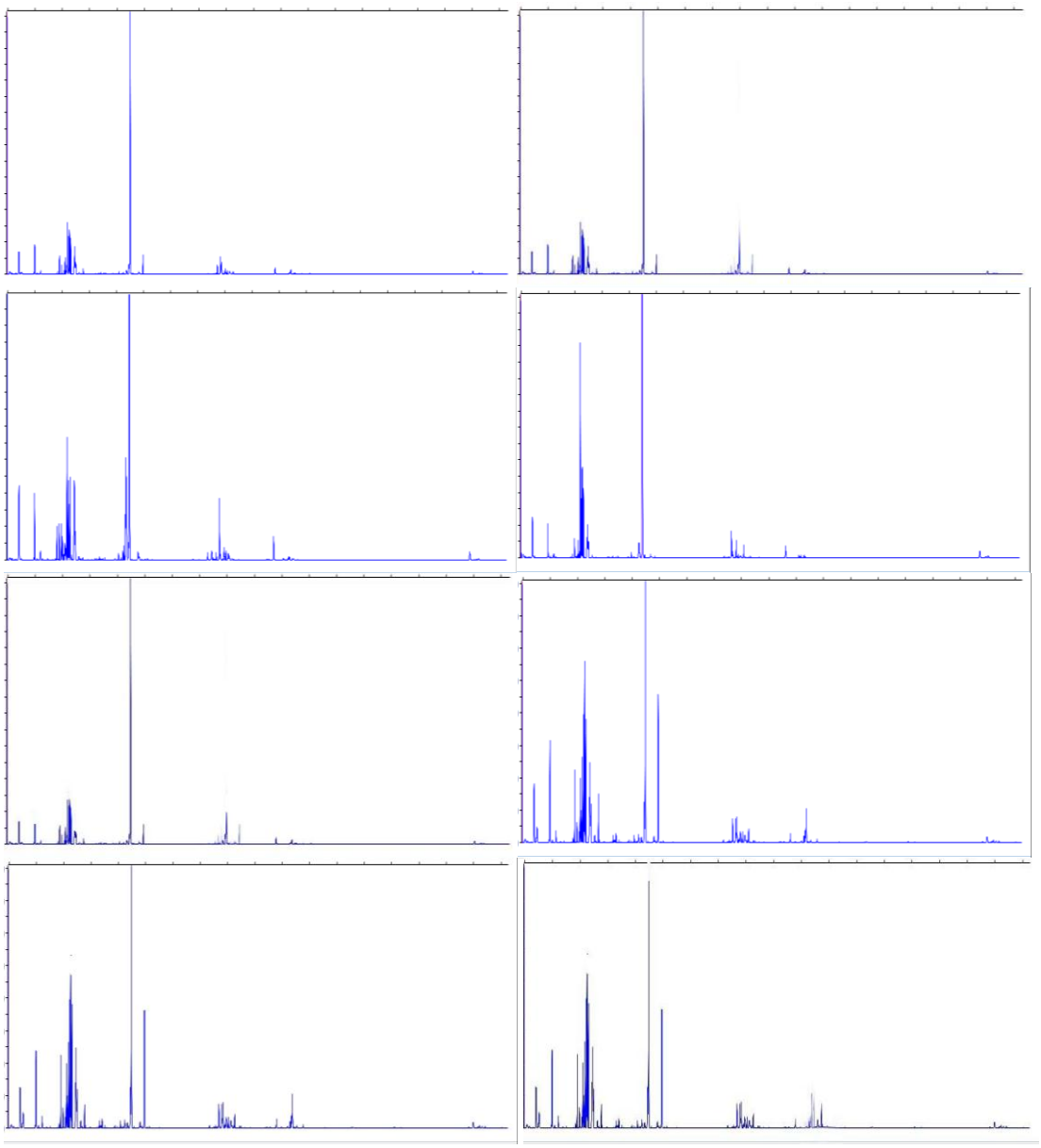


Figure 4.22 Ileum adherent samples of BD+Sr group.

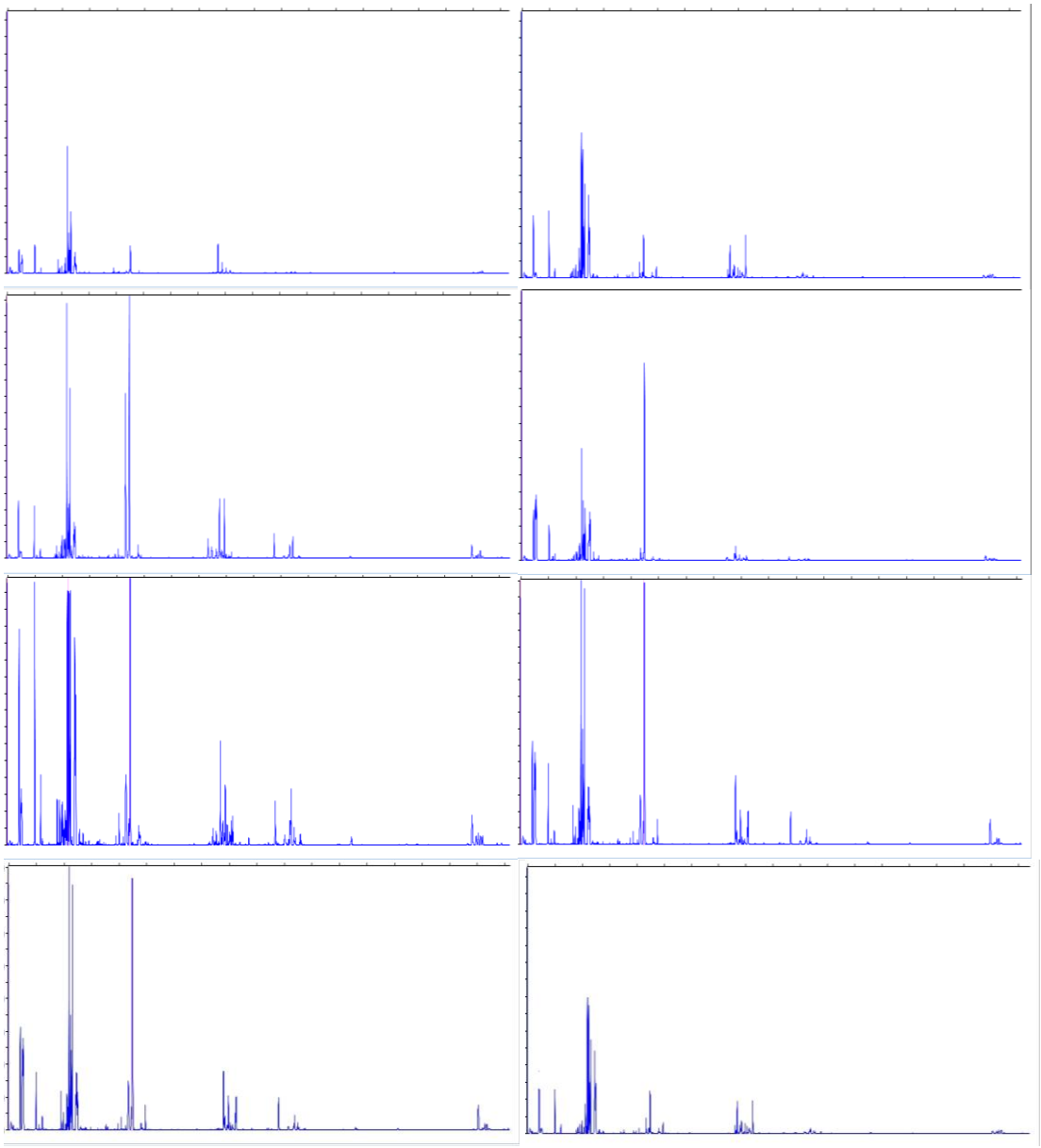


Figure 4.23 Ileum adherent samples of BD+Phy+Sr group.

4.4.2.6 Experiment 2-Ileum adherent digested by Hha I

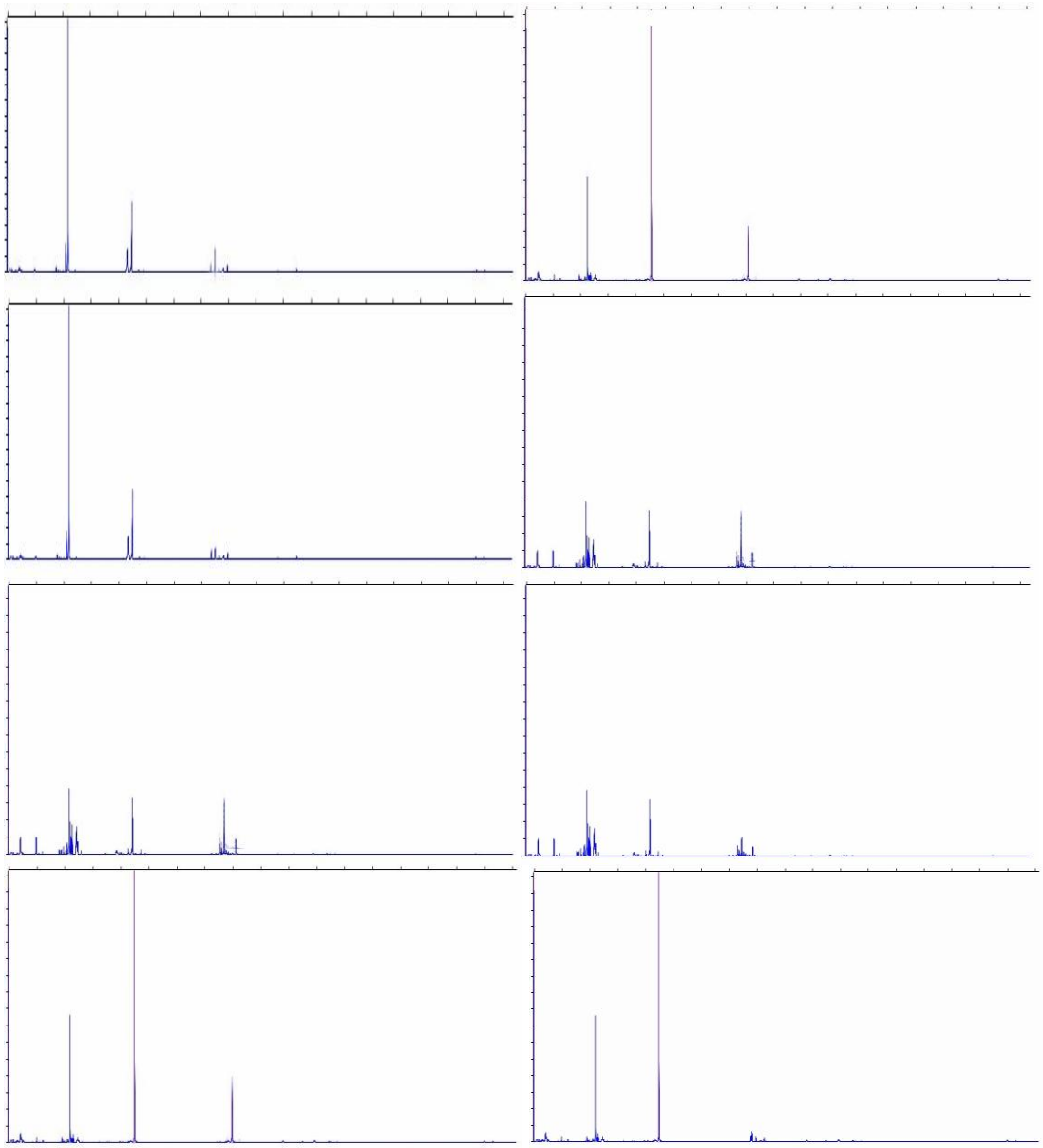


Figure 4.24 Ileum adherent samples of BD group.

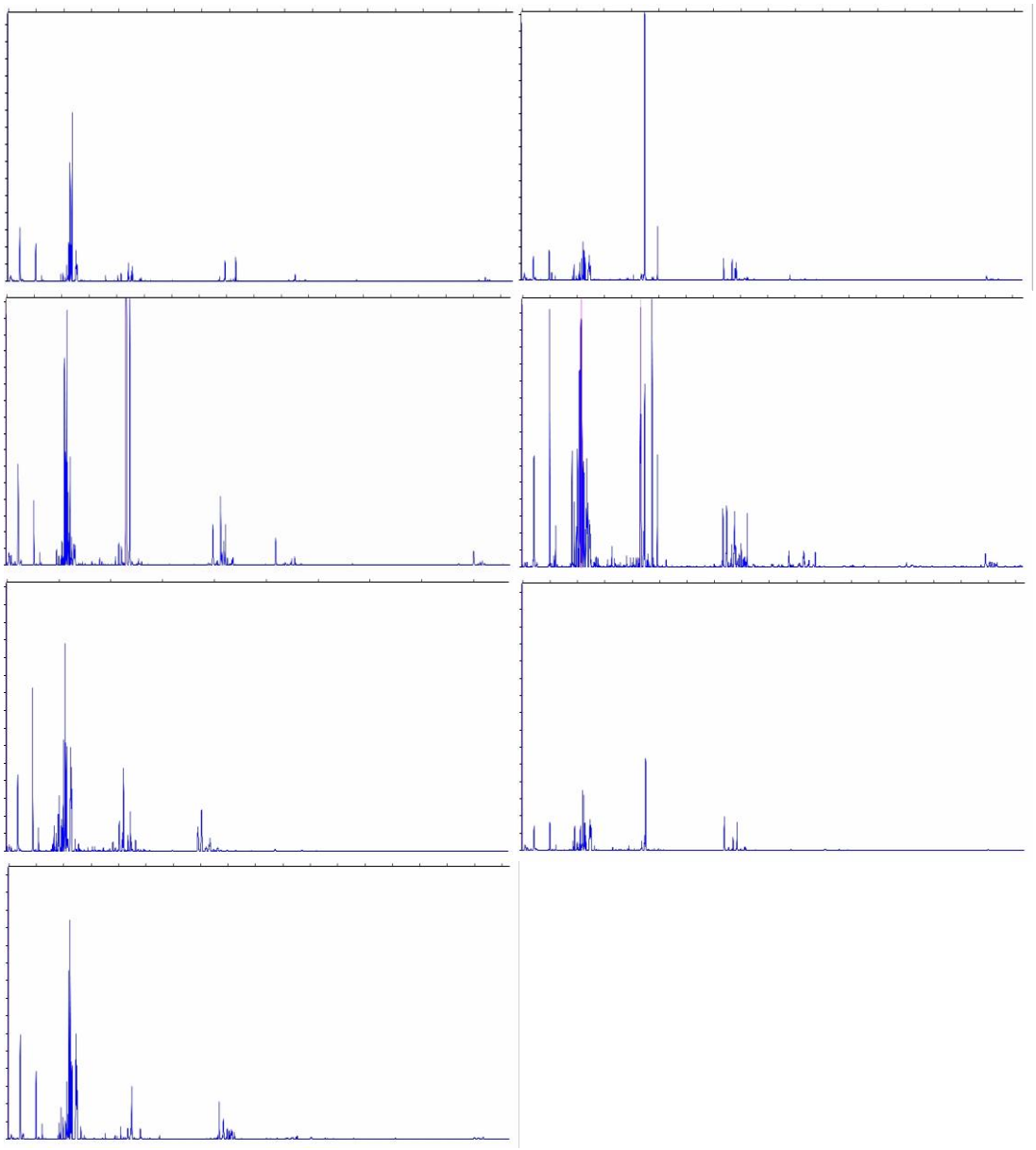


Figure 4.25 Ileum adherent samples of BD+Phy group.

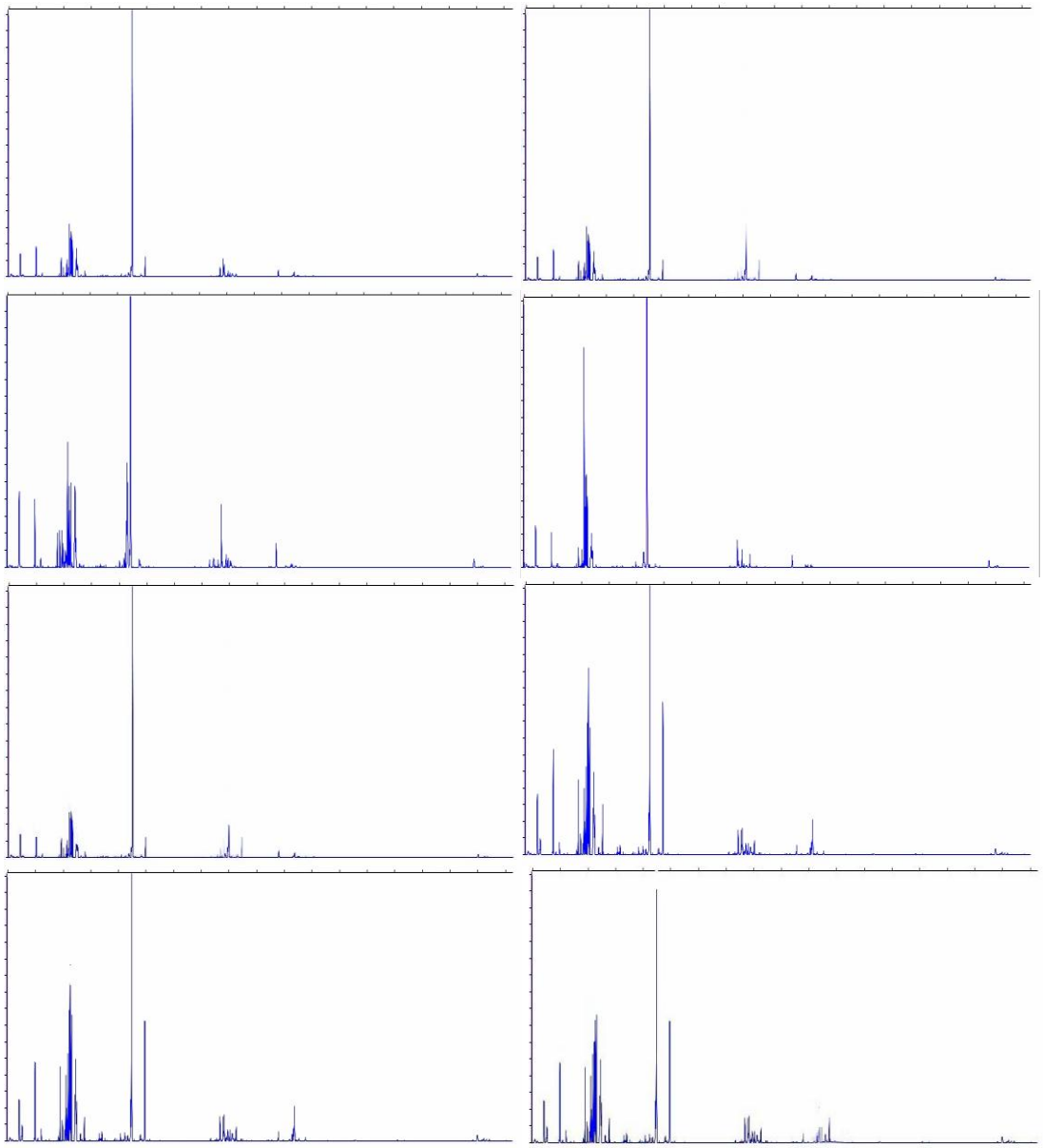


Figure 4.26 Ileum adherent samples of BD+Sr group.

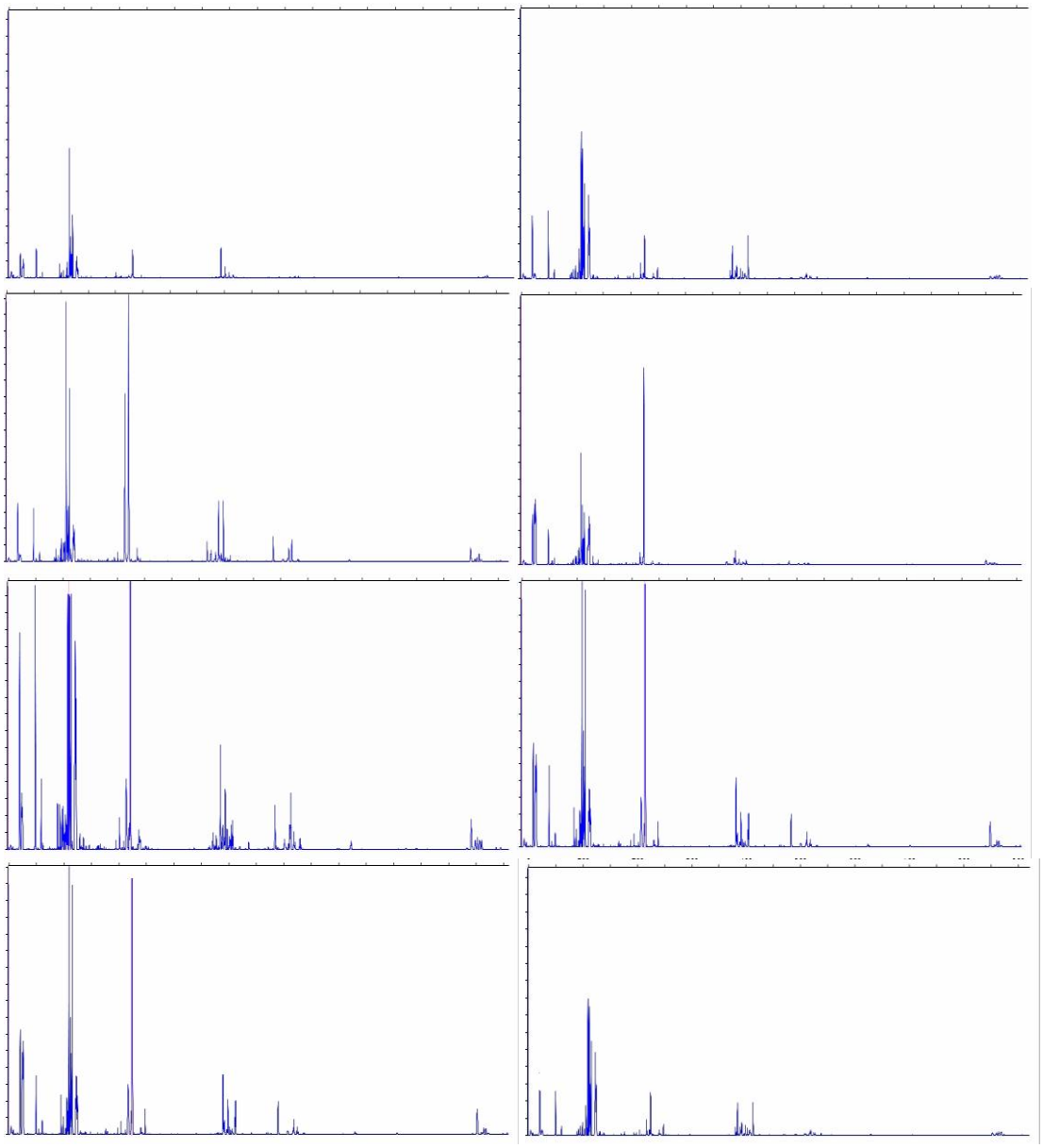


Figure 4.27 Ileum adherent samples of BD+Phy+Sr group.

4.4.2.7 Experiment 2-Mid-colon adherent digested by Bsh 1236I

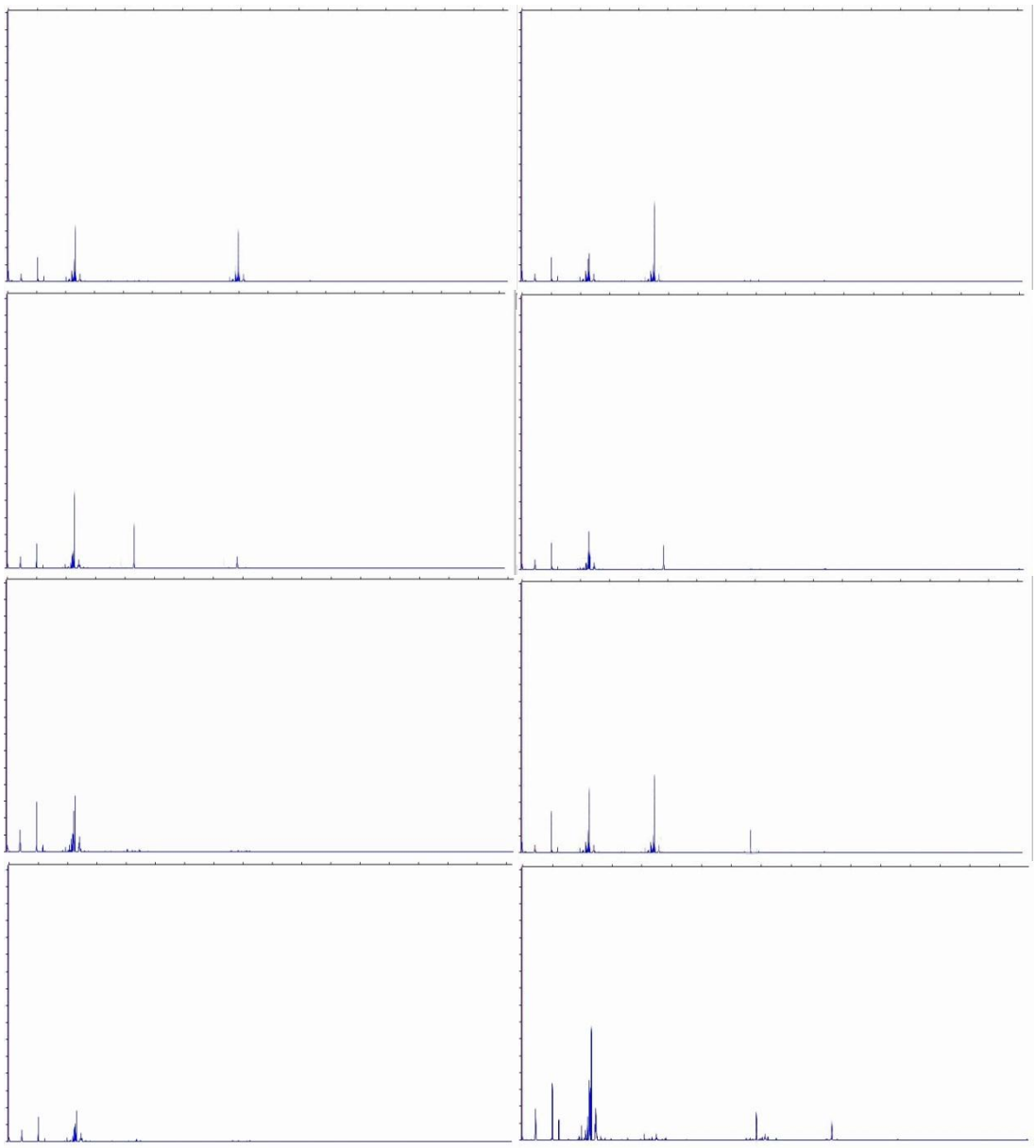


Figure 4.28 Mid-colon adherent samples of BD group.

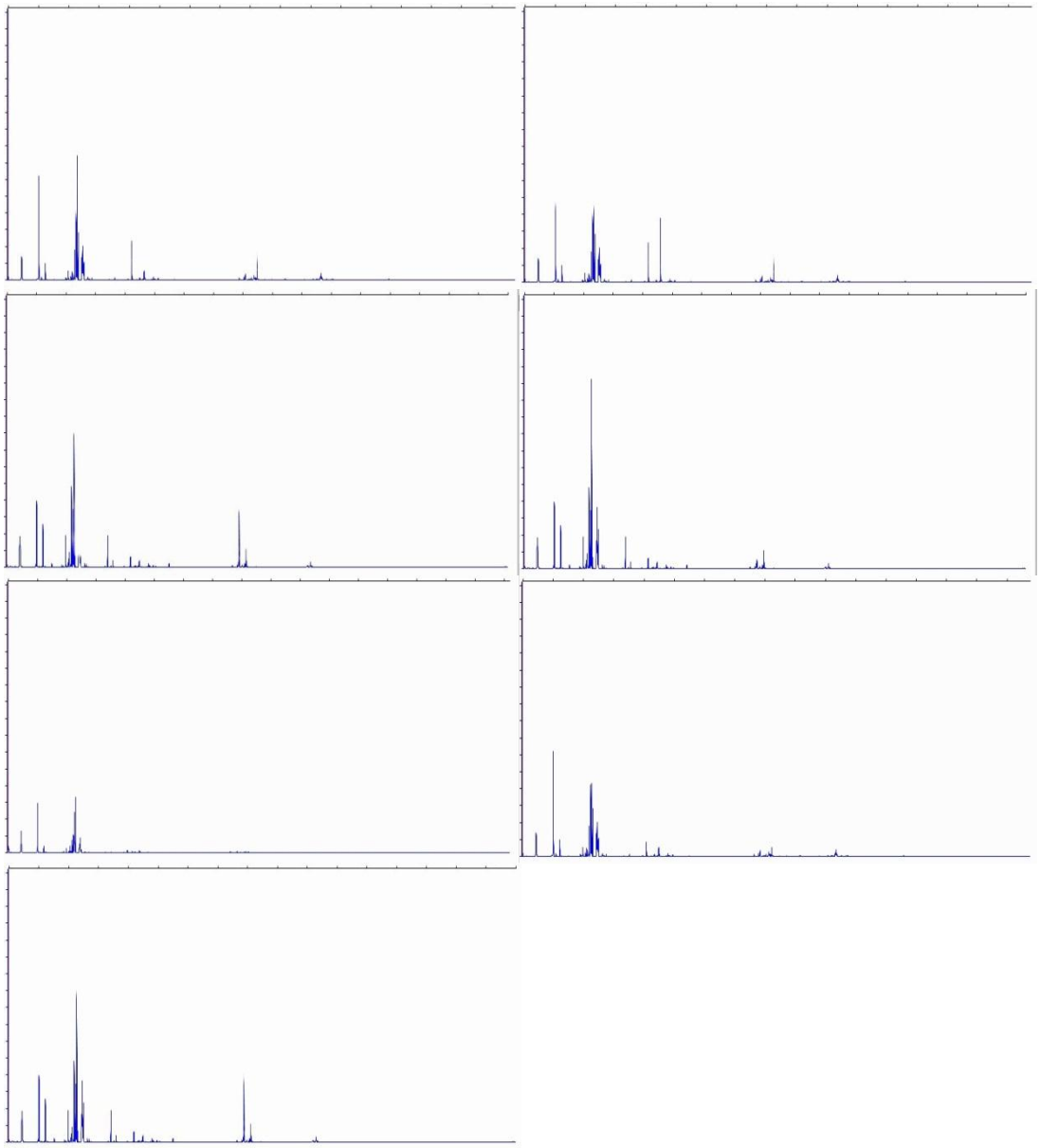


Figure 4.29 Mid-colon adherent samples of BD+Phy group.

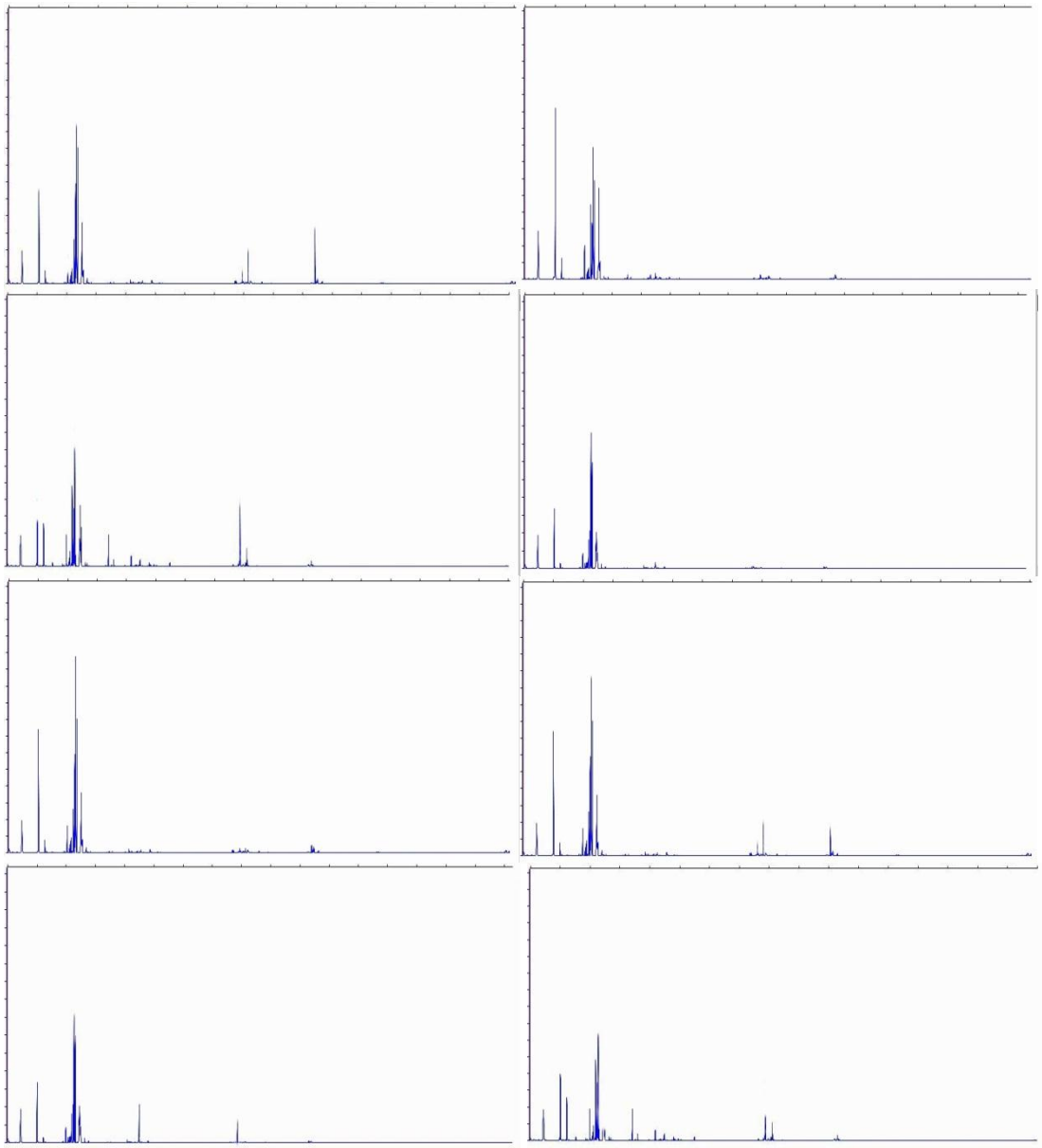


Figure 4.30 Mid-colon adherent samples of BD+Sr group.

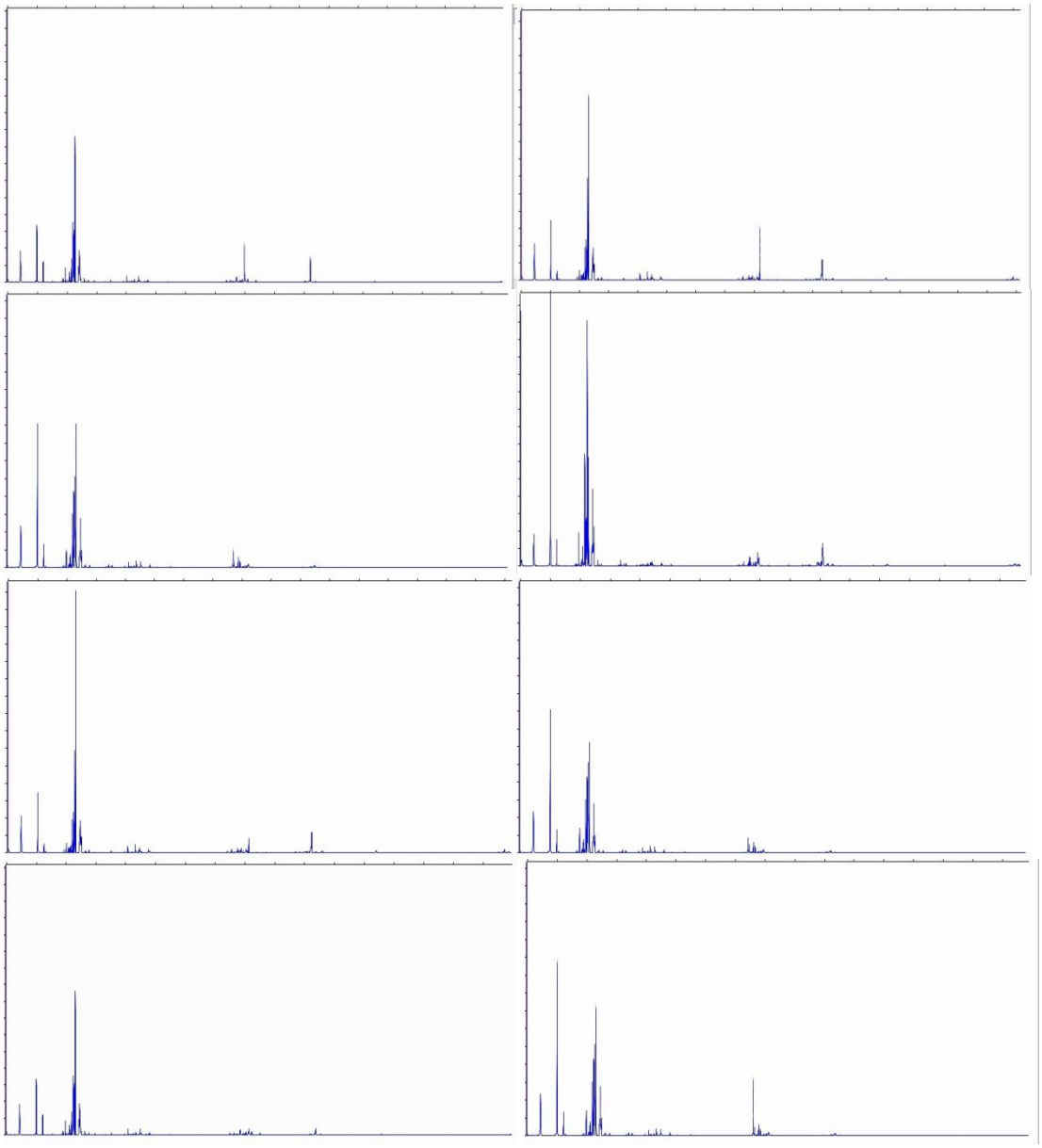


Figure 4.31 Mid-colon adherent samples of BD+Phy+Sr group.

4.4.2.8 Experiment 2- Mid-colon adherent digested by Hha I

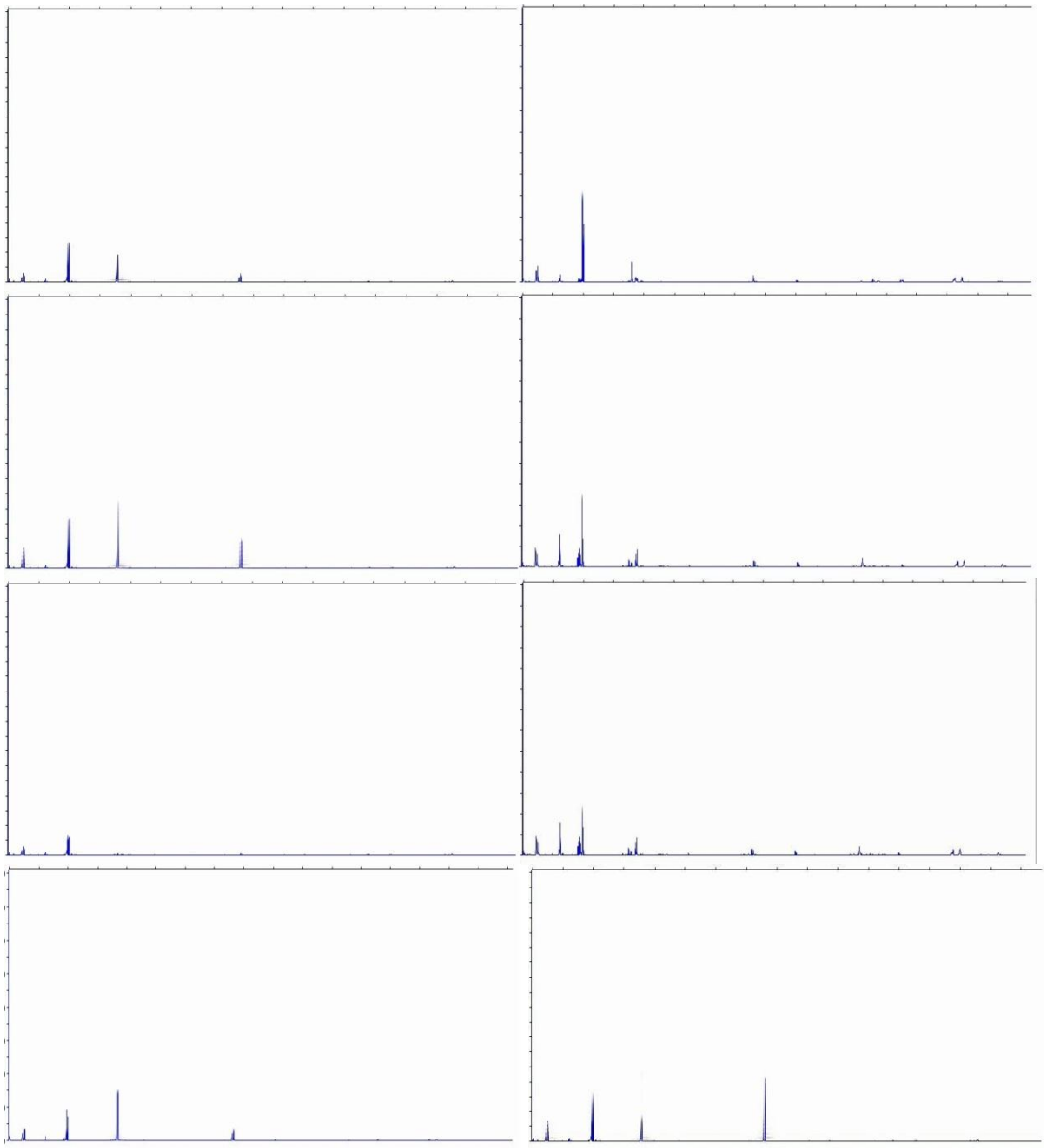


Figure 4.32 Mid-colon adherent samples of BD group.

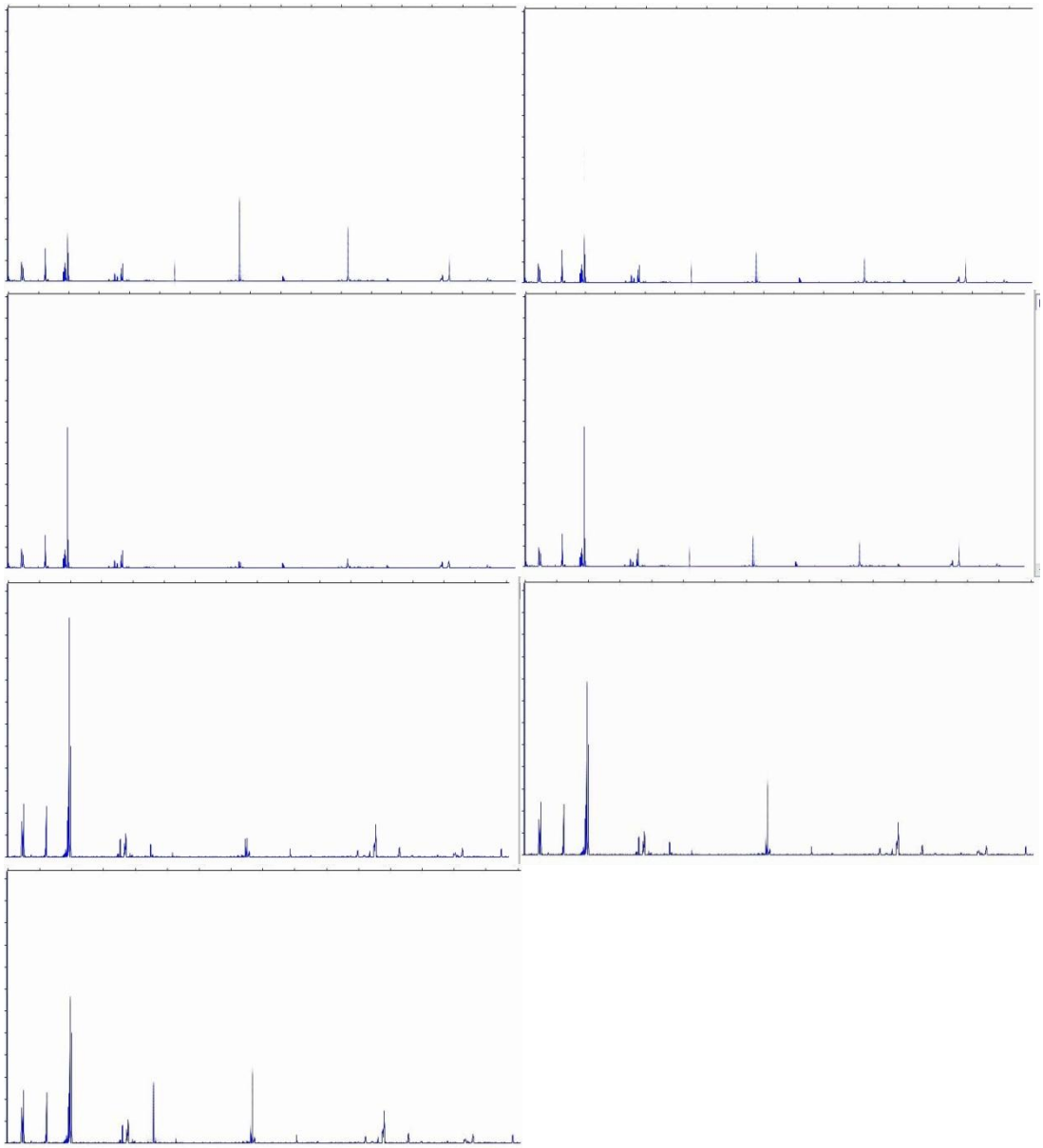


Figure 4.33 Mid-colon adherent samples of BD+Phy group.

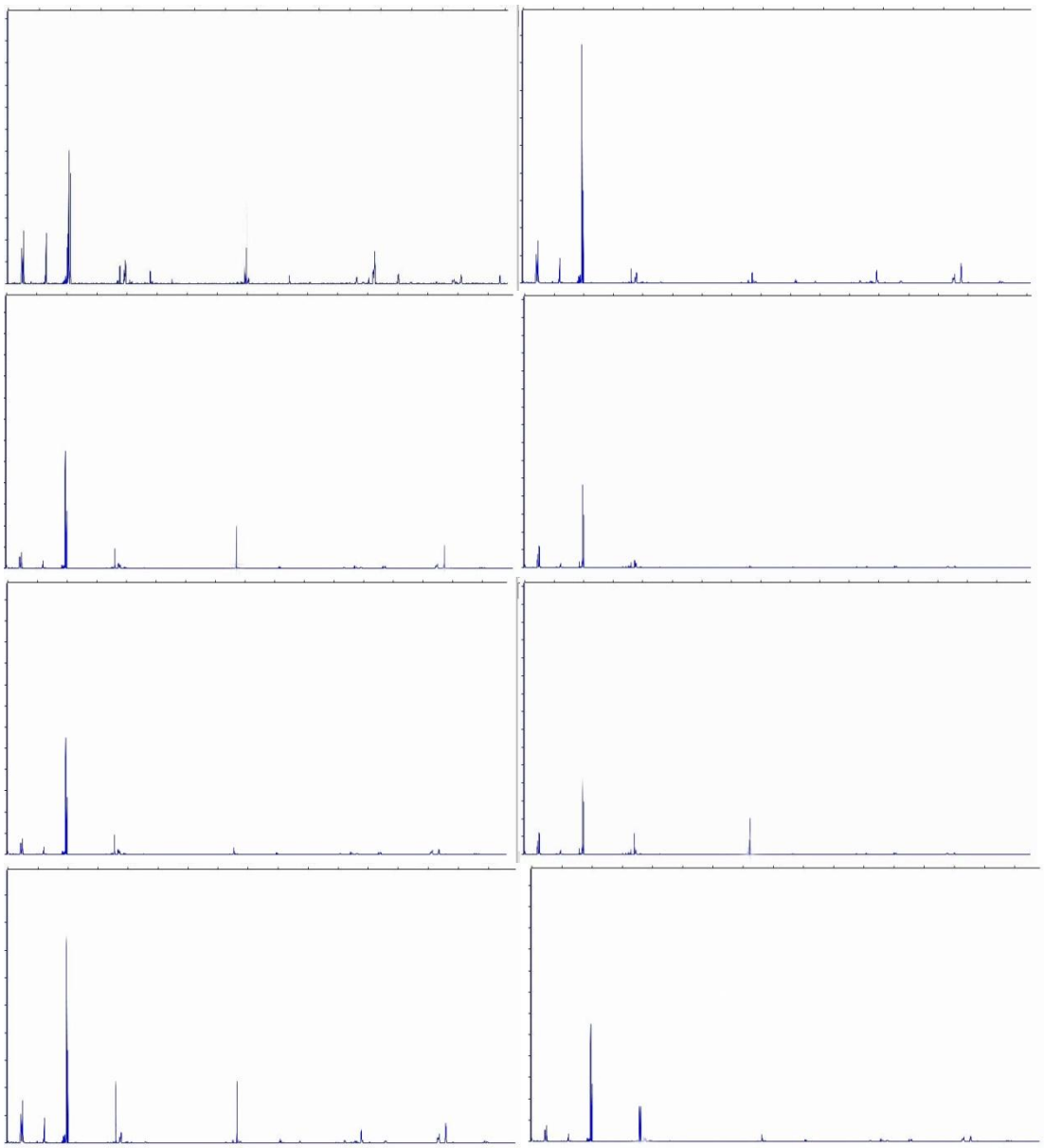


Figure 4.34 Mid-colon adherent samples of BD+Sr group.

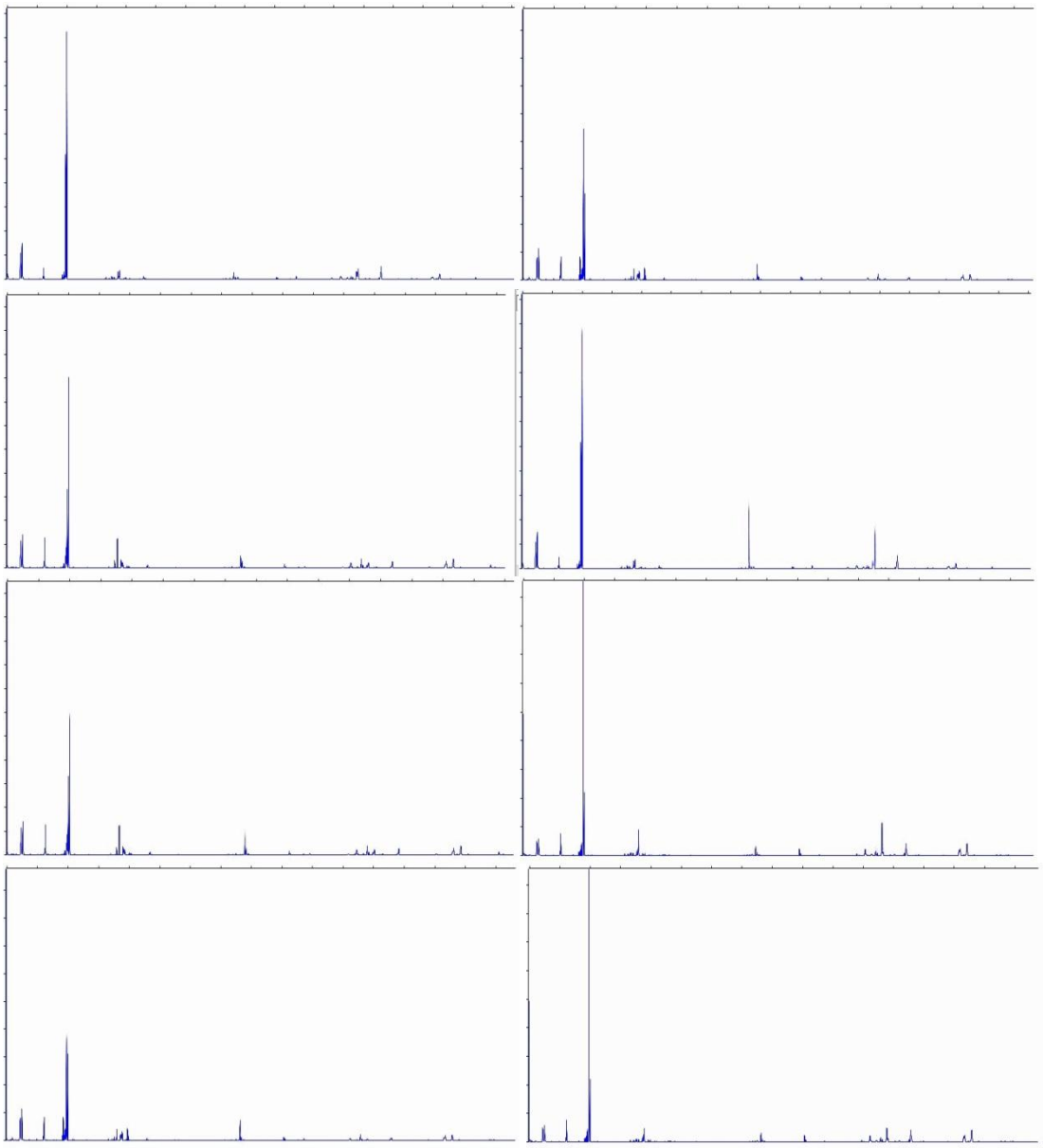


Figure 4.35 Mid-colon adherent samples of BD+Phy+Sr group.

4.4.3 TRFLP results exported from Peak Scanner software: Average Peak length results:

Table4.2 Treatment 1: Ileum samples digested by Bsh1236 I

Experiment1 ileum Bsh	lactobacilli(Proteobacteria)	streptococci/ lactobacilli	clostridium group	E.coli/Samonella(Enterococcus group)
BD	3944.1667	9359.5	4133	937.83333
T1:BD+AppA2	8449.6	12152.2	16254.667	4279.3333
T2:BD+PhyA	9151.2	10443.8	10839	3570.1667

Table4.3 Treatment 1: Ileum samples digested by Hha I

Experiment1 ileum Hha	Bifidobacteria/lactobacilli(Proteobacteria)	clostridium group	E.coli/Samonella(Enterococcus group)	streptococci
BD	2576	2085.666667	8061	6180.333
T1:BD+AppA2	22525.83333	5676.4	10102.66667	20793.5
T2:BD+PhyA	25451.6	5224.4	12062.8	21775.6

Table4.4 Treatment 1: Mid-colon samples digested by Bsh 1236I

Experiment1 colon Bsh	lactobacilli(Proteobacteria)	streptococci/lactobacilli	clostridium group	E.coli/Samonella(Enterococcus group)
BD	2396.8417	4710.17543	1370.8212	15212
T1:BD+AppA2	2748	6527.666667	8776.3333	13941
T2:BD+PhyA	4661.3333	3716.333333	4015	3727

Table4.5 Treatment 1: Mid-colon samples digested by Hha I

Experiment1 colon Hha	Bifidobacteria/lactobacilli(Proteobacteria)	clostridium group	E.coli/Samonella(Enterococcus group)	streptococci
BD	25591.16667	17863.83333	12122.16667	5840.333
T1:BD+AppA2	27336.16667	17325.66667	10764.83333	9373.833
T2:BD+PhyA	20610.16667	10308.66667	13821.5	5577.833

Table4.6 Treatment 2: Ileum samples digested by Bsh1236 I

Experiment2 ileum Bsh	lactobacilli(Proteobacteria)	streptococci/lactobacilli	clostridium group	E.coli/Samonella(Enterococcus group)
BD	4739.3438	6349.493858	6250.2886	9112.5417
T1:BD+Phy	3636.3797	5325.917322	5103.1108	10498.135
T2:BD+Sr	4187.8617	5837.70559	5676.6997	9805.3385
T3:BD+Phy+Sr	4187.8617	5837.70559	5676.6997	9805.3385

Table4.7 Treatment 2: Ileum samples digested by Hha I

Experiment2 ileum Hha	Bifidobacteria/lactobacilli(Proteobacteria)	clostridium group	E.coli/Samonella(Enterococcus group)	streptococci
BD	4739.3438	6349.493858	6250.2886	9112.5417
T1:BD+Phy	3636.3797	5325.917322	5103.1108	10498.135
T2:BD+Sr	4187.8617	5837.70559	5676.6997	9805.3385
T3:BD+Phy+Sr	4187.8617	5837.70559	5676.6997	9805.3385

Table4.8 Treatment 2: Mid-colon samples digested by Bsh1236 I

Experiment2 colon Bsh	lactobacilli(Proteobacteria)	streptococci/lactobacilli	clostridium group	E.coli/Samonella(Enterococcus group)
BD	9371	5148.166667	10462	3660.5
T1:BD+Phy	11337.167	6949.333333	9997.6667	6583.6667
T2:BD+Sr	8720.1667	7054.25641	9789.6667	6294.9744
T3:BD+Phy+Sr	6181	5969	9004.3333	7033.8333

Table4.9 Treatment 2: Mid-colon samples digested by Hha I

Experiment2 colon Hha	Bifidobacteria/lactobacilli(Proteobacteria)	clostridium group	E.coli/Samonella(Enterococcus group)	streptococci
BD	23447.83333	14809.33333	14090.5	5773.5
T1:BD+Phy	30928.5	20224.33333	11276.5	10461.17
T2:BD+Sr	25689.57692	18267.94872	12129.57692	8297.628
T3:BD+Phy+Sr	25593.66667	17048.16667	13583.16667	7408.167

After the PCR step, all of the intestinal adherent samples get about 1.5kb PCR fragments, which is correct length according the universal primers used in this study. All T-RFLP profiles exported from Peak Scanner software and then do the database analysis. The figures exported from Peak Scanner x-axis mean the digested PCR fragments length, which corresponding to adequate bacteria, the y-axis means the unit of the fragments, which express the amount of the relevant bacteria. And according the difference between different treatments, we can have a general idea of how much the feed additives affect the animals. Considering the intestinal microbiota is complex, after comparing with the database, four common and important intestinal bacteria was focus on by this study: lactobacilli(Proteobacteria), clostridium group, E.coli/Samonella group and streptococci.

In experiment 1, from the fragments data of the tables above, the bifidobacteria had a significance ($P<0.01$) increase after feed by both phytases, but there is no difference between two phytase. By contrast, there are no corresponding results of middle colon, difference cannot be seen. Clostridium also show a significant change in two phytase groups, but the PhyA group is more obviously ($P<0.05$, $P<0.01$ respectively). But this phenomenon cannot be observed in middle colon yet. The fragments after digested by Bsh 1236 I and Hha I show different results on the comparison of E.coli/Samonella group, the Bsh 1236 I show a significant change ($P<0.01$) but no difference between two phytase group after treated by Hha I ($P>0.05$), this can be seen both in ileum and middle colon. There is no big difference between two phytase groups on the changing of Streptococci ($P>0.05$) both in ileum and middle colon. Lactobacilli meet the same results of Streptococci.

In experiment 2, both the phytase and strontium changed the population of bifidobacteria in ileum ($P<0.01$), but both of them cannot change the bifidobacteria in middle colon, although there is significant difference between them. Clostridium populatin had been changed in ileum feed by phytase and strontium after the fragments digested by Bsh 1236 I ($P<0.01$), but cannot see the same results in middle colon. Both E.coli/Samonella and Streptococci cannot find any difference among the four feed group in both ileum and middle colon. In this study, both the phytase and strontium changed the population of bifidobacteria in ileum, which indicates that the bifidobacteria play an important role in iron absorption in ileum.

4.5 Discussion

The development of intestinal microbiota of piglets from age 3 week to 9 week was investigated by using 16S rRNA gene-based approaches, focusing on the ileum and middle colon adherent. Changes were observed in GI bacterial community composition of piglets during the period. Two kind phytses from different resource perform the similarity ability on the changing of intestinal microbiota. The bifidobacteria and Clostridium population had been changed significantly by the two phytase. There was no consistent effect of either phytase on any of the detectable bacteria in colon except for that pigs fed AppA2 had slightly higher ($P < 0.05$) bifidobactera content than those fed PhyA. Previous studies on intestinal bacterial microbiota of weaned piglets have shown that E. coli concentrations increased while numbers of lactobacilli decreased after weaning [27, 29, 30], in our study, total bacteria population markedly increased, of which the percentage of bifidobacteria and Clostridium increased significantly.

Colon is usually to be considered the most important site colonized of microbiota, our study shows that ileum is more susceptible to feed additives and the microbiological population changed more often.

The previous study shows the positive effect of low-level Sr supplementation on bone breaking strength and material properties extends our knowledge of this element on bone metabolism. In this study, it is the first time to provide some information about the relationship of intestinal health and bone metabolism.

Despite there are some disadvantages of T-RFLP to be the only method applied in this research, we can also get an integrated link of how these feed additives affect the gut health. But further researches are needed to quantity the microbiota population and amount. Gene cloning and sequencing are necessary step.

In conclusion, AppA2, PhyA phytases and stontium showed a stronger impact on microbiota in ileum than in colon. Our finding reveals a novel function of phytase beyond nutrition.

4.6 Conclusions

Phytate is the major storage form of phosphorus in seeds and so is a common dietary constituent. According to previous study, feed adequate dose phytase had significant effects on feed intake, growth performance, Plasma P status, but there have been no previous studies illustrating how much phytase can affect the intestinal microbiota.

4.7 References

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CHAPTER 5

Effects of Sodium Benzoate on Intestinal Microbiology of Weaned Piglets

5. Effects of Sodium Benzoate on Intestinal Microbiology of Weaned Piglets

5.1 Abstract

The aim of this investigation was to evaluate the efficacy and tolerance of Sodium Benzoate in weaned piglets fed mixed diet. The effect of Na-benzoate on the intestinal microflora in piglets was also investigated.

Ninety-six (96) piglets weaned at 24 d, weight averaging 7.40 kg, were assigned to three treatments groups, Control (C), Na benzoate 0.4% (T1) and Na benzoate 3.6% (T2). Piglets' health, growth performance and microbiological parameters were monitored.

At the end of trial, piglets of T1 group had higher weight at the end of the trial than Control (P= 0.056) and T2 group (P< 0.0001). Piglets from Control were heavier than piglets fed diet supplemented with 3.6 % Sodium benzoate (P= 0.015).

ADG was higher in T1 than T2 group during all the trial (P=0.002), whereas growth of Control piglets was higher than T2 group from 0 to 14 d postweaning (P<.0001). Piglets fed 0.4 % Na benzoate also showed higher ADFI (P= 0.041) and a better FCR (P= 0.067) than Control. Piglets fed 3.6 % Na benzoate had also higher ADFI than Control group during starter phase (P= 0.0001) and overall (P= 0.006).

Both Na benzoate dietary supplementation determined a significant reduction of total aerobes, total anaerobes, Streptococci, and Enterobacteriaceae at different time. Clostridia faecal concentration was lower at day 3 (T1) and day 42 (T2).

5.2 Introduction

Organic acid has been used for many years for improving animal performance and to prevent digestive problems. The addition of organic acid generally lowers the pH and increases GI acidity, promotes beneficial bacteria and decrease pathogens. A study was shown that feeding benzoic acid to weaning pigs improved piglets' performance and was associated with a greater ileal microbiota biodiversity [30]. Some of the most used organic acidifiers include formic, acetic,

propionic, butyric, lactic, sorbic, fumaric, malic, and citric acid, Ca-formate, Ca-lactate, Ca-propionate, K-diformate, Ca-butyrate, Mg-citrate and Na-lactate.
The study objective is to evaluate the efficacy and tolerance of Sodium Benzoate in weaned piglets fed mixed diet and the effect of Na-benzoate on the intestinal microflora in piglets.

5.3 Materials and methods

Two completely randomised block (room) design with 3 experimental treatments (Table 3). Each treatment is replicated 8 times with 4 piglets/pen (replicate) forming the experimental unit.

Table 5.1 Experimental Design

Treatment	Sodium Benzoate	
	Prestarter (24 d of age)	Starter (38-65 d of age)
Ctr	-	-
T1 Sodium Benzoate 0.4 %	4 kg/ton	4 kg/ton
T2 Sodium Benzoate 3.6 %	36 kg/ton	36 kg/ton
Number of treatments: 3	Piglets per replicate: 4	
Replicates (pens) per treatment: 8	Piglets per treatment: 32	
Total number of replicates: 24	Total number of piglets: 96	

5.3.1 Experimental animals and housing

The trial was carried out at the facility of Centro Zootecnico Didattico Sperimentale, Polo di Lodi, Università degli Studi di Milano, Italy. In the nursery barns, the animals were housed in a environmental controlled nursery room, containing 24 pens. Each pen is slatted and measures 1.50 m x 1.25 m. The house is lit by a combination of daylight (through skylights) and artificial light (non-programmable). Ventilation is achieved by single, variable-speed fans linked to temperature sensors. The temperature inside the building was approximately 28ºC at the start of the trial, adjusted weekly until a final temperature of 20 ºC. Non-pelleted diets were provided ad libitum throughout the trial from hoppers and water from 2 nipple drinkers per pen.

Piglets were obtained from a single weaning and were weaned at 24 d, weights averaging 7.40 kg. A total of 96 crossbreed piglets were allocated according to body weight to the experimental treatments. A randomisation process was used

to allocate piglets to replicates, such that each treatment consists of an equal number of homogeneous replicates (with regard to weight). Piglets were placed in 24 replicates (pens of 4 piglets).

5.3.2 Experimental diets

All diets were calculated to be nutritional equivalent, and meet or exceed the nutrient requirements recommended by NRC (1998) for pigs. The composition and the calculated analysis of the diets according to FEDNA (2003) are presented in Table 4. Diets were manufactured in the feed mill of FAMAVIT Feed Company (Brescia, Italy), the appropriate quantity of Sodium Benzoate was premixed with barley before addition to the final mix to ensure a homogeneous distribution in the final feed. Feed was in mash form. In order to prevent cross-contamination, control feed was produced first followed by production of the feeds supplemented with Sodium Benzoate. All diets were prepared without the inclusion of any feed acid, antibiotic, or AGPA - antibiotic growth promoter alternative (e.g. herbs, spices, essential oils, high Cu/Zn). Care was taken to ensure feed homogeneity, especially in relation to fat addition. The feeds were made before the start of the trial, to allow time for nutritional homogeneity and analysis. Feeds were stored in a cool dry place until required. Feeds were analyzed for nutritional homogeneity (crude protein, crude fiber, ether extract, starch, ash and moisture; AOAC, 2000) by the feed analysis laboratory of Dept. Veterinary Sciences and Technologies for Food Safety. Parallel feed samples are analyzed for Sodium benzoate by Benzoic acid content in the feeds.

5.3.3 Feed sampling and analysis

- Diet samples. Representative samples (500 g) from all trial diets were taken after manufacturing of the feeds.

- Proximate analysis of the diets: moisture (dry matter), nitrogen (crude protein), ether extract, crude fibre, starch and ash (local laboratory), and Sodium benzoate analysis.

- Faeces samples. Representative samples from all piglets were taken at 0, 3, 7, 14, 28, 42 days on trial. Samples from piglets of each pen were pooled, refrigerated and immediately transported to the laboratory to be analyzed for total anaerobes and aerobes, Lactobacilli, Enterobacteriaceae, Clostridia, Streptococci , Salmonella spp and Campylobacter spp.

5.3.4 Performance measurements/observations

- Performance. Piglets were weighed individually at 0, at 14 and at 42 days on trial (end of trial). Feed intake was recorded per replicate at the same time as the weighing. Performance parameters (daily gain, feed intake and feed efficiency) were determined.

- The veterinarian checked the piglets when they were put on trial. Only healthy piglets were allowed to the trial, and no prophylactic antibiotic treatment was given.

- The consistency of faeces was assessed at 0, 14, 42 days in every pen (1 normal, 2 soft, 3 diarrhea).

- Mortality/culling were recorded: just one piglet (control group) died during the trial.

5.3.5 Measures to minimize risk of cross contamination

Control animals were always attended first followed by animals in the Kemira groups. Risk was reduced by carefully cleaning boots and other relevant equipment that might be contaminated. Special care was taken when weighing piglets at mid-trial and at 42 d of age.

5.3.6 Statistical analysis of the data

The data were analysed as a completely randomised block design by proc mixed of SAS v. 6.12 (SAS Institute, 1990). Data is presented as least square means by periods and for the complete trial.

Table 5.2. Composition and calculated analyses of the experimental diets[1]

Ingredients, %	Prestarter 1-14 d			Starter 15-42 d		
	C	T1	T2	C	T1	T2
Micronized Wheat	13	13	13	-	-	-
Micronized Maize	12.5	12.5	12.5	10	10	10
Micronized Barley	17.6	17.6	17.6	10	10	10

Barley	10.04	9.8	6.6	24	24	24
Maize	-	-	-	12.34	12.1	8
Extruded soybean seed	-	-	-	8	8	8
Wheat	-	-	-	10	10	10
Whey soluble (80% lactose)	11.5	11.5	11.5	7	7	7
Skimmed Milk	7	7	7	2.5	2.5	2.5
Soycomil	5	5	5	2.5	2.5	3.2
Herring meal	6.8	6.8	6.8	5.8	5.8	5.8
Protein potato	2.5	2.5	2.5	1.8	1.8	1.8
Full Fat whey	6	6	6	-	-	-
Pig Lard	3.8	3.8	3.8	1.8	1.8	2
Dicalcium phosphate	0.9	0.9	0.9	0.6	0.6	0.6
Calcium Carbonate	0.5	0.5	0.5	0.85	0.85	0.85
Lysine HCl 78	0.67	0.67	0.67	0.65	0.65	0.65
DL Methionine	0.32	0.32	0.32	0.29	0.29	0.29
L-Threonine	0.27	0.27	0.27	0.28	0.28	0.28
L-Tryptophan	0.11	0.11	0.11	0.1	0.1	0.1
Premix[1]	1.33	1.33	1.33	1.33	1.33	1.33
Salt	0.16	-	-	0.16	-	-
Sodium benzoate	--	0.4	3.6	--	0.4	3.6
Calculated analyses, % as fed						
Dry matter, %	89.7	89.7	89.7	88.5	88.5	88.5
DE, kcal/kg	3900	3900	3900	3560	3560	3560
Crude protein, %	19.5	19.5	19.5	18.5	18.5	18.5
Crude fibre, %	1.75	1.75	1.75	2.75	2.75	2.75
Ether extract, %	7.75	7.75	7.75	5.4	5.4	5.4
Total lysine, %	1.51	1.51	1.51	1.4	1.4	1.4
Total SAA, %	0.9	0.9	0.9	0.84	0.84	0.84
Threonine, %	0.97	0.97	0.97	0.91	0.91	0.91
Tryptophan, %	0.29	0.29	0.29	0.27	0.27	0.27
Calcium, %	0.69	0.69	0.69	0.67	0.67	0.67
Total phosphorus, %	0.52	0.52	0.52	0.51	0.51	0.51
Sodium, %	0.30	0.30	0.82	0.26	0.26	0.78

[1] Vitamin-mineral premix supplies per kg final feed: Vitamin A: 10,500 IU; Vitamin D3: 2,500 IU; Vitamin E: 15 mg; Vitamin B1: 1.5 mg; Vitamin B2: 3.8 mg; Vitamin B12: 0.025 mg; Vitamin B6: 1.6 mg; Calcium pantotenate: 12 mg; Nicotinic acid: 15 mg; Biotin: 0.15 mg; Folic acid: 0.5 mg; Vitamin K3: 3 mg; Fe: 100 mg; Cu: 6 mg; Co: 0.75 mg; Zn: 150 mg; Mn: 65 mg; I: 0.75 mg; Se: 0.4 mg; Ethoxyquin: 150 mg.

5.4 Results and discussion

5.4.1 Growth performance

The effects of feeding Na benzoate on piglets' performances are shown in the table 5.

Table 5.3 Growth performance

	Control	T1 0.4%	T2 3.6%	SE	P		
					CxT1	CxT2	T1xT2
Weight, kg							
Start	7.39	7.35	7.39	0.36	0.943	0.993	0.936
Day 12	12.17	12.41	11.28	0.36	0.639	0.081	0.026
Day 42	27.19	28.17	25.94	0.36	0.056	0.015	<.0001
ADG, g/d							
Days 0-12	399	422	324	12.4	0.188	<.0001	<.0001
Days 12-42	501	525	489	12.4	0.159	0.491	0.035
Days 0-42	471	496	441	12.4	0.167	0.087	0.002
ADFI, g/d							
Days 0-12	464	493	455	21.9	0.351	0.775	0.225
Days 12-42	749	829	879	21.9	0.014	0.0001	0.112
Days 0-42	668	733	758	21.9	0.041	0.006	0.423
FCR							
Days 0-12	1.17	1.17	1.41	0.02	0.942	<.0001	<.0001
Days 12-42	1.49	1.58	1.80	0.02	0.019	<.0001	<.0001
Days 0-42	1.41	1.48	1.72	0.02	0.067	<.0001	<.0001

Piglets from T1 group had higher weight at the end of trial than Control (P= 0.056) andT2 group (P< 0.0001), while Control piglets had better weight than T2 Piglets at the end of the trial (P=0.015).

ADG was higher in piglets fed 0.4 % Na benzoate than those fed 3.6 % Na benzoate at any time, whereas ADG of piglets fed control diet was higher than T2 group only during the prestarter phase (P<.0001). Piglets from T1 group also showed higher ADFI and better FCR than Control group during the starter phase (P= 0.014, P= 0.019 respectively) and overall period (P= 0.041, and P= 0.067 respectively). FCR was also better in T1 than T2 group during all trial (P< 0.0001).

Piglets fed the highest dose of Na benzoate had higher ADFI than Control group during the starter phase (P= 0.0001) and overall period (P=0.006).

5.4.2 Health parameters

The effects of feeding Na benzoate on health parameters are shown in the table 6. A daily check was carried out during the whole trial, in order to record all possible incidents and implement suitable measures. During the trial, one piglet from the control group died (pneumonia). No significant differences among the groups were observed in fecal score.

Table 5.4: health parameters

	Ctr Control	T1 0.4%	T2 3.6%	SEM	*P < F*		
					CxT1	CxT2	T1xT2
Faecal score [1]							
Start	1.25	1.25	1.12	0.15	1.00	0.56	0.56
Day 14	1.25	1.12	1.25	0.15	0.56	1.00	0.56
Day 42	1.12	1.12	1.00	0.15	1.00	0.56	0.56
Piglets dead/eliminated[2]	1	--					

[1] Faecal score scale: 1 normal faeces; 2 soft faeces; 3 diarrhoea
[2] One piglet died for pneumonia (control group)

5.4.3 Microbiological determinations in faeces

The microbiological counts in faeces are presented in table 7. Sodium benzoate dietary supplementation significantly reduced number of total aerobes, total anaerobes, Enterobacteriaceae and Streptococci at different times. Clostridia faecal concentration was lower in piglets fed Na benzoate at day 3 (T1) and at day 42 (T2). Lactobacilli resulted lower in piglets fed 0.4 % Na benzoate (day 28), and in piglets fed the highest dose (days 7, 14, 28 and 42). No piglets from all treatment groups were Salmonella and Campylobacter positive.

Table5.5 Microbiological counts

	C control	T1 0.4 %	T2 3.6 %	SE	P		
					CxT1	CxT2	T1xT2
Total aerobes, *log$_{10}$ CFU/g*							
Day 0	7.96	7.65	8.18	0.16	0.18	0.35	0.02
Day 3	8.03	7.71	7.81	0.16	0.18	0.36	0.66
Day 7	8.20	7.87	7.27	0.16	0.16	0.001	0.01
Day 14	8.52	7.74	7.61	0.16	0.001	0.002	0.59
Day 28	8.22	7.76	7.22	0.16	0.05	<.0001	0.02
Day 42	7.44	7.90	6.55	0.16	0.09	0.001	<.0001
Total anaerobes, *log$_{10}$ CFU/g*							
Day 0	8.27	7.93	8.47	0.16	0.13	0.37	0.02
Day 3	8.47	8.23	8.22	0.16	0.27	0.26	0.97
Day 7	8.68	8.55	7.65	0.16	0.57	<.0001	<.0001
Day 14	8.85	8.22	7.82	0.16	0.005	<.0001	0.08
Day 28	8.59	8.17	7.56	0.16	0.06	<.0001	0.01
Day 42	8.07	8.03	7.14	0.16	0.85	<.0001	0.0001
Streptococci, *log$_{10}$ CFU/g*							
Day 0	6.03	5.49	5.66	0.30	0.20	0.38	0.69
Day 3	5.41	5.00	4.66	0.30	0.34	0.08	0.43
Day 7	3.90	4.12	3.07	0.30	0.61	0.05	0.01
Day 14	3.99	3.76	2.98	0.30	0.59	0.02	0.07
Day 28	3.70	3.48	3.01	0.30	0.62	0.11	0.27
Day 42	3.12	2.68	2.10	0.30	0.31	0.02	0.18
Enterobacteriaceae, *log$_{10}$ CFU/10g*	7.86	7.43	8.05	0.40	0.44	0.74	0.27
Day 0	7.69	7.29	5.75	0.40	0.48	0.0009	0.01
Day 3	7.33	7.07	2.09	0.40	0.65	<.0001	<.0001
Day 7	6.80	5.87	7.02	0.40	0.10	0.71	0.05
Day 14	6.72	5.88	6.44	0.40	0.14	0.62	0.32
Day 28	6.57	5.97	5.53	0.40	0.29	0.07	0.43
Day 42							
Clostridia, *log$_{10}$ CFU/g*							
Day 0	5.57	4.24	4.81	0.26	0.0005	0.04	0.13
Day 3	5.13	4.02	4.48	0.26	0.003	0.08	0.21
Day 7	3.00	3.00	3.00	0.26	1.00	1.00	1.00
Day 14	0.00	0.50	0.00	0.26	0.17	1.00	0.17
Day 28	0.25	0.25	0.50	0.26	1.00	0.50	0.50
Day 42	0.29	0.54	1.12	0.26	0.50	0.02	0.11
Lactobacilli, log$_{10}$ CFU/g							
Day 0	7.59	7.42	8.22	0.29	0.69	0.13	0.06
Day 3	7.83	7.56	7.84	0.29	0.51	0.98	0.50
Day 7	8.29	7.97	6.78	0.29	0.44	0.0004	0.005
Day 14	8.71	7.96	7.17	0.29	0.07	0.0004	0.06
Day 28	8.38	6.99	7.00	0.29	0.001	0.001	0.98
Day 42	7.54	8.28	6.42	0.29	0.08	0.01	<.0001

Salmonella							
Day 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Campylobacter							
Day 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Day 42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

5.5 Conclusions

Post-weaning dietary supplementation with 0.4 % Na benzoate resulted in higher growth performance. Moreover, it determined a significant reduction of total aerobes, anaerobes, and Clostridia. No detrimental effect was observed in piglets fed diet supplemented with the highest dose of Na benzoate.

5.6 References

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CHAPTER 6

General Discussion

6. General discussion

The main aim of the research described in this thesis was to provide new insights of three kinds of non-antibiotic feed additives into the response of piglet's gastrointestinal (GI) tract microbiota to in-feed supplementation. Pig gut microbiota is a complex ecosystem, which can be changed during different growth period and affected by different feed composition. The unbalancing intestinal microbial levels and post-weaning GI tract disorders can cause important economic losses in the pig industry. The ban on the antibiotics use in the European Union started from 2006 requires us to find new alternatives to overcome the issue.

Modulation of the microbial composition and activity is possible by change the feed composition. Plant resource additives have been used more often in recent years. They have been recognized as rich and diverse sources of a wide range of different bioactive ingredients of potential use in animal production, including oligosaccharides and prebiotic dietary fibers, as well as a broad range of additional bioactive compounds that can be extracted from plant.

phytase supplementation has been effectively used to improve utilization of phytate-P in diets of pigs and poultry reducing their fecal P excretion and releasing phytatechelated minerals for absorption by these species. Considering the gastrointestinal tract is the most important site for absorption, there is necessary to take a view of the relationship between phytase and GI microbiota. Organic acids supplementation in piglet feed has been a common practice for enhancing animal performance and to prevent digestive problems, especially in the post- weaning period for a long time. But there is few studies take attention of the how the organic acid change the animal growth performance, this research may supply a new way for deep research.

CHAPTER 7

Summary

7. Summary

This thesis presents the study focus on the response of piglet GI tract microbiota afer the supplementation with different feed additives, combine with different research methods. This research work is focus on the relationship between feed additives and intestinal health. The gastrointestinal (GI) tract of pigs is colonized by a variety of active microbiota which shows the symbiotic relationship with host. Also the gastrointestinal tract microbiota plays such an important role in pig health. Supplementing pigs with different diet ingredients that can have an effect on the GI tract microbiota is a common strategy to affect pig's health. In this study, in order to detect the non-antibiotic anti-microbial effect of nutritional additives, all the three trials show three different resource additives using variety tecniques.

This study consists of three independent trials; all the feed additives used in this study are non-antibiotic aim to increase the intestinal anti-microbial effect. The application of different techniques in this thesis show the anti-microbial effect of different non-antibiotic feed additives which provided important information of following research works.

CHAPTER 8

Acknowledgements

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