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EC MICROBIOLOGY
Research Article

Bacillus lentus Metabolites with Antimicrobial Activity as a New Generation of Growth Promoters for Animals

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Received: February 27, 2020; **Published:** April 28, 2020

Abstract

Bacteriocin-like inhibitory substances produced by a new strain *Bacillus lentus* B-7150 have shown a significant effect on broiler production in the Markinskaya research farm (B-1207, Russia), demonstrating high potentialities of getting benefits from their use. It is shown that providing broiler chickens with feed along with *B. lentus* bacteriocin-like substance has significantly stimulated broiler production. So, the live weight of 35 day-old broilers increased by 20.2% and 19.1%, with average daily weight gain increasing by 20.6% and 19.7%, while feed conversion decreased by 4.08% and 6.62% compared to additives-free and flavophospholipol-supplemented controls, respectively. Possible ways of stimulate growth are discussed.

Keywords: *Bacteriocin; Bacillus lentus (B. lentus)*

Introduction

Unlike antibiotics bacteriocins and Bacteriocin Like Inhibitory Substances (BLIS) show no toxic or negative effects and are considered as ecologically friendly additives as probiotics and phytobiotics without restrictions for their use with feed. BLIS and other biologically active substances (BAS) were produced from soil *Bacillus lentus* strain B-7150. We have produced samples of the BLIS-containing substance from *B. lentus* (BLIS-BL) for testing them with feed on animals. Experiments were carried out on farm broilers and lasted totally 35 days.

Objectives of the Study

- Derive main biologically active substances from *Bacillus lentus* strain B-7150.
- Prepare samples containing *Bacillus lentus* - derived antimicrobials to treat broilers.
- Prepare broilers for further trials.
- Determine broiler weights, feed consumption and some other selected parameters.
- Process obtained results and discuss them.

Methods

The strain of bacilli was culture at 30°C for 48 hours. Technology of producing coarse fractions of these substances for testing in figure 1. Bactericidal activity was determined by placing samples of desired volumes in Petri dishes containing freshly seeded lawns of test strains of Gram-positive and Gram-negative microorganisms. The activity was expressed in arbitrary units (AU) measured for 1 ml or 1 mg of the sample depending on the level of dilution. The method of two-phase separation in the presence of organic solvent (dichloromethane, chloroform) was found to be the most effective one to isolate the product from the culture fluid [1]. Molecular identification of the bacteriocin-like substance was performed by SDS PAGE [2]. Before biological testing the gel was washed, placed in a Petri dish and overlaid with melted agar containing test cells. Molecular weight of the sample determined by MALDI-TOF Bruker Daltonics mass spectroscopy. Experiments with broilers were performed in accordance with the methodological recommendations for conducting research on the feeding of poultry.

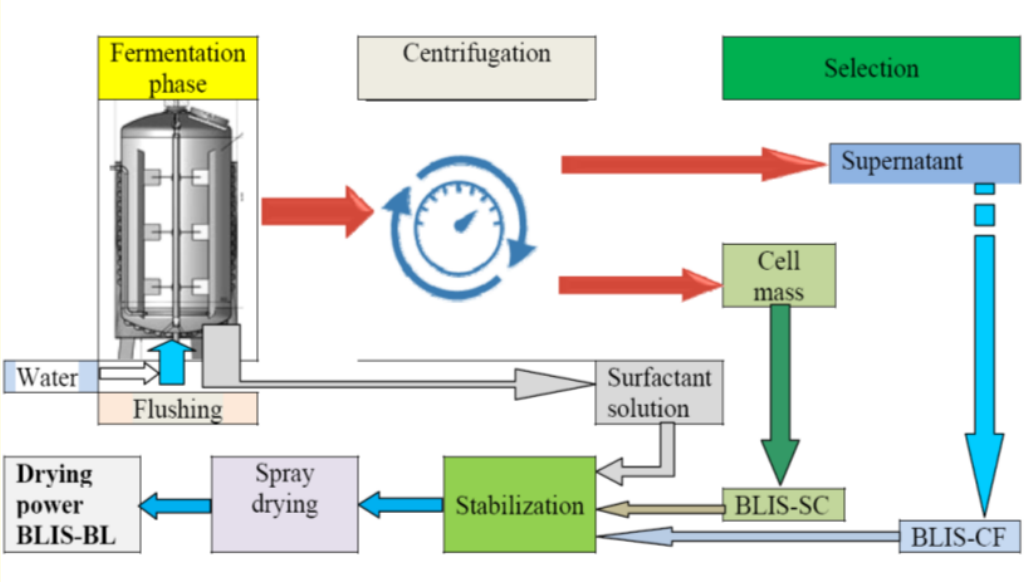

Figure 1: *BLIS-BL production scheme.*

The study of the microflora of the blind processes and litter: After slaughtering the chickens by cutting off the head, 0.5 - 0.7g of the contents of the blind processes, litter samples were taken and introduced into sterile tubes with 4.5 ml of isotonic NaCl solution. A series of 10-fold dilutions was prepared with initially diluted (7 - 10 times) suspensions of each sample. The resulting dilutions were sown on several types of culture media: universal purpose (fish meal hydrolyzate-based nutritional agar, manufacturer SRCAMB), selectively differential nutrient (Levina-, Endo-, bismuth-sulfite-, entero- and staphylococci- Agar, from SRCAMB; Perfringens Agar Base, Campylobacter Agar, MRS agar, HiMedia), and chromogenic culture media (XLD Agar, M1393 and M1295, HiMedia), followed by culturing at 30 - 37°C for 24 - 48 hours. The study of cell morphology was carried out Gram stain microscopy.

Results and Discussion

***B. lentus* biologically active substances**

- BSF - biosurfactant (≈900 Da) active against Gram(-) bacteria (Figure 2).
- BLIS from the cell surface (BLIS-SC) (< 10 kDa) targeting mainly Gram(+) bacteria (Figure 3b).
- BLIS from the cell-free culture fluid (BLIS-CF) ≈4 kDa targeting mainly Gram (-) bacteria (Figure 3a).
- Alkaline protease is of special interest due to its ability to inactivate both its own and foreign bacteriocins. Production of the enzyme are nearly concurrent with the dynamics of antimicrobial activity (See figure 4a).

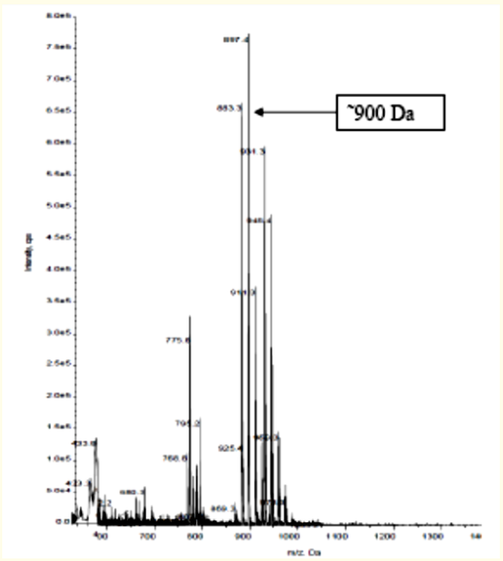

Figure 2: *Molecular weight of the B. lentus biosurfactant sample as determined by MALDI-TOF Bruker Daltonics mass spectroscopy.*

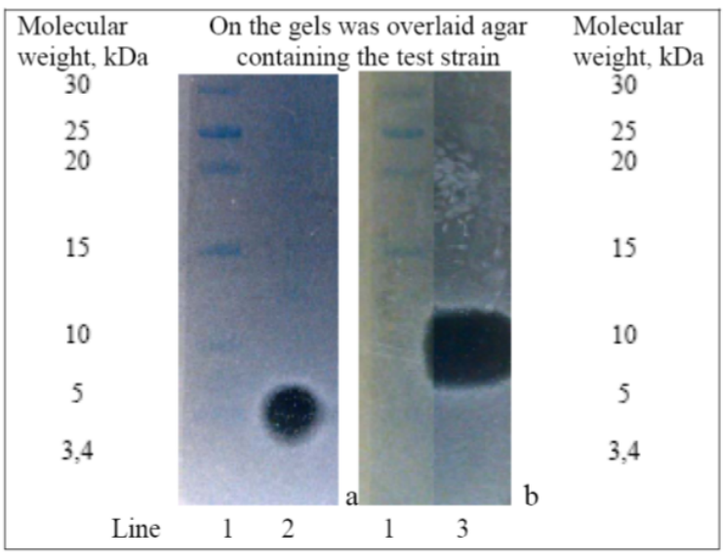

Figure 3: *SDS PAG Electrophoresis of B. lentus BLIS samples. Line: 1 - marking PageRuler™, 2 - BLIS-CF, 3 - BLIS-SC.*

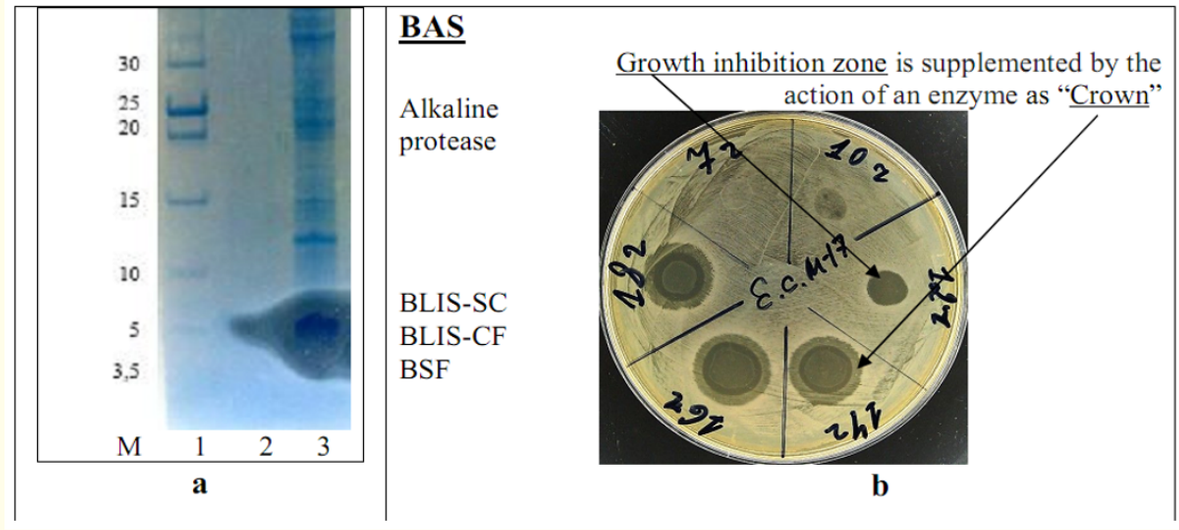

Figure 4: *Molecular weights (lanes) of B. lentus substances as determined by SDS PAG electrophoresis (a); antimicrobial effect of the substances on the growth of the test-strain; visualized influence of the enzyme on the medium (b). M - marking, kDa; № 2, 3 - BAS.*

Subject and conditions of trials

- The effect of BLIS-BL on broiler production was evaluated. The activity unit is an Arbitrary Unit (AU), with 1 kg of dry BLIS being equal to 1,000,000 AU.
- The dosage of BLIS-BL of approximately 1,000 AU/kg of complete feed that is equal to 900g of this BLIS per one ton of feed used in the trial.
- What we expected from BLIS-BL application: antimicrobial action (especially against *Clostridia perfringens, Campylobacter jejuni, Salmonella* spp. and *E. coli*) and harmonization of intestinal microflora.
- Broilers (males) Ross-308 were used; 80 broilers/one treatment (4 replica, 20 broilers/pen, floor penning - see figure 5).

Figure 5: *Distribution of birds in poultry farms Hall.*

- Measurements: Live weight of broilers, feed intake.
- Floor litter and cecal samples were taken after the trial for microbiological analysis.
- 100 ml of manure were taken from each pen and homogenized for further analysis.
- For comparison with BLIS-BL (T7) we used conventional premixes: antibiotic (T2), probiotics (T3-T6), organic acid (T8), copper sulfate (T9) served as a control forage without additive T1.

Conclusion

- Strain *B. lentus* B-7150 produces several biologically active substances, properties and conditions of which have been explored [3]. BLIS-SC adsorbs on the surface of cells and is released by means of polar solvents. BLIS-CF and alkaline protease are released into the interphase film under influence of non-polar solvents. Biosurfactant attaches to the walls of the bioreactor and can be removed with hot water with alkaline pH. Solutions BLIS and protease are stabilized with polyvinylpyrrolidone followed by spray-drying (Figure 1). Then dry powder is normalized to obtain the specific antimicrobial activity. The dry mix is added to broiler feed.
- Trials have shown that (1) the live weight of 35- day-old broilers increased by 20.2 and 19.1 percent, (2) the average daily gain weight was 20.6 and 19.7%, (3) feed conversion decreased by 4.08 and to 6.62% *vs* additive-free and flavophospholipol-added controls, respectively (Figure 6 and table 1-4).
- Unlike antibiotics bacteriocins and BLIS produce neither toxic nor negative effects and are considered as ecologically friendly additives as probiotics and phytobiotics without restrictions for their use with feed.
- No significant changes in bacterial profiles of broiler ceca or litter samples were observed. It was also shown that BLIS-BL is not harmful for useful *E. coli* and *Enterococcus faecium* (Table 5). However, this question should be clarified.
- It quite possible that intestinal toll-like receptors are involved in these actions [4,5]. This effect of bacterial cells and their separate components has been actively studied in medicine, especially in pediatrics.
- Alkaline protease acts from jejunum throughout colon and ceccum, masking digestion more efficient. Production of the enzyme are nearly concurrent with the dynamics of antimicrobial activity (See figure 4).
- This effect of bacterial cells and their separate components is widely investigated in human medicine, especially in pediatrics.

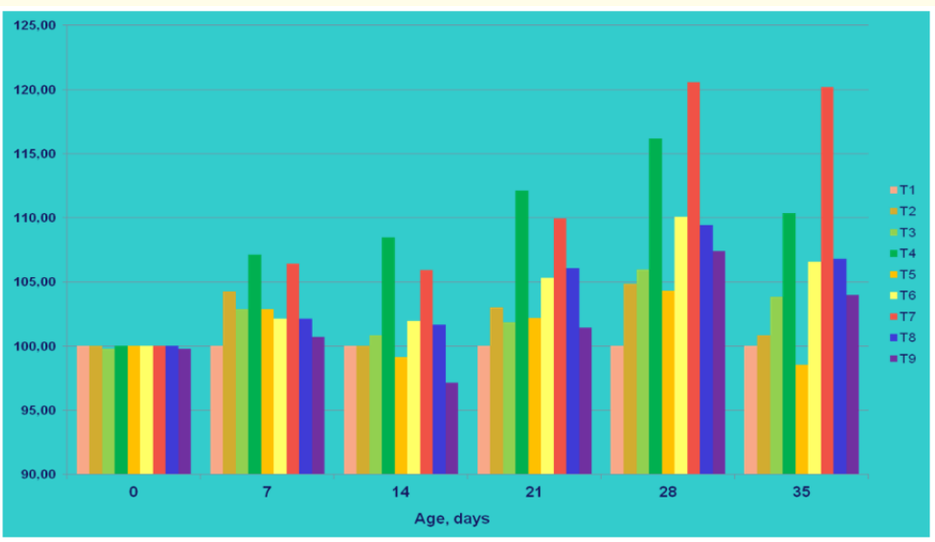

Figure 6: *Dynamics of gain weight by broilers provided with BLIS-added (T7) feed compared to the other groups.*

Days	Control diet (CD)	CD + flavopho-spholipol	Probiotics				BLIS BL	Organic acid	Copper sulfate	Mean square deviation (MSD)	P-value
	T1	T2	T3	T4	T5	T6	T7	T8	T9		
0	46.3	46.3	46.2	46.3	46.3	46.3	46.3	46.3	46.2	0.18	0.9996
7	140 b	146 ab	144 ab	150 a	144 ab	143 ab	149 ab	143ab	141 b	1.77	0.0093
14	354 c	354 c	357 bc	384 a	351 c	361 bc	375 ab	360 bc	344 c	4.03	0.0001
21	693 c	714 c	706 c	777 a	708 c	730 bc	762 ab	735 abc	703 c	9.15	0.0001
28	1070 c	1122 c	1134 bc	1243 ab	1116 c	1178 bc	1290 a	1171 bc	1149 bc	23.1	0.0001
35	1504 bc	1517 bc	1562 bc	1660 ab	1482 c	1603 bc	1808 a	1606bc	1564 bc	34.65	0.0001

Table 1: *Body weight at different weighing dates.*

Days	T1	T2	T3	T4	T5	T6	T7	T8	T9	MSD	P-value
0 - 7	13.44 b	14.18 ab	13.95 ab	14.85 a	13.95 ab	13.84 ab	14.61 ab	13.81 ab	13.5 b	0.25	0.0079
8 - 14	30.5 bc	29.7 c	30.4 bc	33.4 a	29.5 c	31.0 abc	32.4 ab	31.0 bc	29.0 c	0.49	0.0001
15 - 21	48.4 c	51.4 abc	49.8 bc	56.0 a	51.0 abc	52.8 abc	55.3 ab	53.6 abc	51.3 abc	1.17	0.0018
22 - 28	54.0 c	58.3 bc	61.1 bc	66.7 ab	58.4 bc	64.0abc	75.5 a	62.3 bc	63.7 abc	2.58	0.0005
29 - 35	61.9	56.5	61.0	59.4	52.2	60.7	73.9	62.1	59.3	3.98	0.0713
0 - 35	41.7 bc	42.0 bc	43.3 bc	46.1 ab	41.0 c	44.5 bc	50.3 a	44.6 bc	43.4 bc	0.99	0.0001

Table 2: *Average daily gain in the different periods of growing.*

Days	T1	T2	T3	T4	T5	T6	T7	T8	T9	MSD	P-value
0 - 7	1.507	1.530	1.547	1.452	1.522	1.530	1.512	1.540	1.530	0.024	0.2978
8 - 14	1.482	1.582	1.572	1.512	1.616	1.540	1.510	1.515	1.640	0.036	0.074
15 - 21	1.752	1.717	1.755	1.695	1.720	1.747	1.750	1.682	1.712	0.042	0.9119
22 - 28	2.262 a	2.087 ab	1.942 bc	1.862 bc	2.092 ab	1.930bc	1.700 c	1.967abc	1.917 bc	0.064	0.0002
2 9- 35	1.580	1.825	1.705	1.707	1.940	1.640	1.757	1.782	1.740	0.106	0.4843
0 - 35	1.762 ab	1.810 ab	1.745 ab	1.692 b	1.852 a	1.725 b	1.690 b	1.757 ab	1.755 ab	0.026	0.0041

Table 3: *Feed conversion rate in the different periods of growing.*

Days	T1	T2	T3	T4	T5	T6	T7	T8	T9	MSD	P-value
0 - 7	142	152	151	151	149	148	155	149	144	3.1	0.2008
8 - 14	316	329	335	354	334	335	343	330	334	8.97	0.2995
15 - 21	592	617	798	836	803	825	833	811	819	17.78	0.7071
22 - 28	848	845	830	870	856	861	899	855	853	20.86	0.5852
29 - 35	674 b	718 b	713 b	691 b	701 b	698 b	909 a	777 b	716 b	26.82	0.0001
0 - 35 days total	2571 b	2662 b	2640 b	2729 b	2653 b	2687 b	2980 a	2740b	2662 b	45.41	0.0002

Table 4: *Feed intake in the all period of growing.*

Trials	The number of dominant microorganisms per 1 g of fecal mass											
	Total number of aerobes	*E. faecalis*	*E. faecium*	*Staph.* spp.	*E. coli*	Other coliforms	*Salm.* spp.	*Bac.* spp.	*Proteus* spp.	Microaerophiles and anaerobes		
										Lb. spp.	*Cl. perfringens*	*C. jejuni*
1	$5,9.\ 10^6$	$2,1.\ 10^4$	$4,4.\ 10^5$	$2,8.\ 10^3$	$1,6.\ 10^5$	$1,4.\ 10^5$	$1,5.\ 10^3$	$1,1.\ 10^5$	HO	$4,8.\ 10^7$	ND *	ND
2	$4,1.\ 10^6$	$4,0.\ 10^4$	$3,5.\ 10^5$	$3,0.\ 10^6$	$5,2.\ 10^6$	$1,7.\ 10^6$	$1,9.\ 10^2$	$5,0.\ 10^6$	HO	$1,4.\ 10^7$	ND	ND
3	$1,3.\ 10^7$	HO	$3,3.\ 10^5$	$6,6.\ 10^4$	$5,0.\ 10^5$	$3,3.\ 10^5$	$2,4.\ 10^3$	$4,6.\ 10^4$	$1,2.\ 10^2$	$3,1.\ 10^7$	ND	ND
4	$4,4.\ 10^6$	$2,2.\ 10^5$	$4,6.\ 10^5$	$3,7.\ 10^2$	$3,2.\ 10^5$	$4,4.\ 10^5$	$1,1.\ 10^3$	$2,3.\ 10^3$	HO	$1,1.\ 10^7$	ND	ND
5	$5,6.\ 10^6$	$1,1.\ 10^5$	$8,7.\ 10^5$	$1,4.\ 10^3$	$4,5.\ 10^5$	$1,3.\ 10^5$	HO	$1,7.\ 10^2$	$1,5.\ 10^2$	$3,6.\ 10^7$	ND	ND
6	$7,8.\ 10^5$	$6,8.\ 10^4$	$1,4.\ 10^5$	$8,4.\ 10^4$	$7,2.\ 10^4$	$9,4.\ 10^2$	$1,4.\ 10^3$	$5,0.\ 10^2$	$2,1.\ 10^2$	$1,2.\ 10^6$	ND	ND
7	$7,6.\ 10^6$	$1,4.\ 10^5$	$1,3.\ 10^6$	$1,1.\ 10^4$	$8,5.\ 10^5$	$7,6.\ 10^5$	$2,2.\ 10^3$	$1,3.\ 10^5$	HO	$1,5.\ 10^7$	ND	ND

Table 5: *Data analysis of the microbial composition of the contents of the blind processes.*
(*): Not Detected; Bac.: Bacillus; Lb.: Lactobacillus; Cl.: Clostridium; C: Campylobacter.

Acknowledgments

We thank personnel of the Markinskaya poultry farm and the SRCAMB for invaluable contribution to the research.

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Volume 16 Issue 5 May 2020